REMARKS

Claims 1-109 are pending after entry of this response. Claim 1 has been allowed. Claims 67-79 and 84-108 have been withdrawn from consideration. Claims 2-66, 80-83, and 109 remain rejected. Applicants acknowledge the allowance of claim 1.

Reconsideration and withdrawal of the following rejections under 35 U.S.C. §112, first paragraph are respectfully requested.

Response to Rejections under 35 U.S.C. §112, First Paragraph

Claims 2-66, 80-83 and 109 have been rejected under 35 U.S.C. §112, first paragraph for allegedly failing to comply with the written description requirement. Specifically, the Examiner brings forth the Federal Register Notice (Vol 66 FR 1099, Friday, January 5, 2001) which discusses guidelines for examination of patent applications under 35 U.S.C. §112, first paragraph, written description requirement in view of *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Based on this case, the Examiner alleges that the instant application does not disclose structural features and properties commonly shared and possessed by each of the claimed genus of cellulases, genus of endoglucanases and a genus of cellobiohydrolases (Office Action – page 4). Furthermore, the Examiner alleges that the scope of each genus includes many members with widely differing structural, chemical, and physicochemical properties including widely differing amino acid sequences and there is no correlation between these structures with their respective enzymatic activities. Thus, according to the Examiner, one skilled in the art cannot predict, visualize, and/or recognize the identity of

the members of each claimed genus. Applicants respectfully disagree with the Examiner for the following reasons.

With respect to the Examiner's contention that cellulases, endoglucanases and cellobiohydrolases are separate genera (Office Action – page 3), applicants assert that the art recognises one genus of cellulases. According to Henrissat, et al. (see attached Henrissat, et al., *Gene*, 81(1):83-95, Sept. 1, 1989), the cellulase genus consists of "[s]ix families of cellulases ... identified on the basis of primary structure homology," whereas endoglucanases and cellobiohydrolases fall within the genus of cellulases. Moreover, applicants describe in the instant specification numerous cellulases that can be further subclassified as endoglucanases and cellobiohydrolases. See for example pages 54-68 of the instant specification, Example 12 – Purification of Cellulase Components, which describes various purification techniques. Therefore, endoglucanases and cellobiohydrolases should be considered as further subgroups of cellulases and should not be treated as separate genera.

The Examiner contends that there are no disclosed structural features and properties commonly shared and possessed by the claimed genus of cellulases. Applicants respectfully disagree and direct the Examiner's attention to the fact that a cellulase under basic definition is an enzyme that catalyzes chemical reactions (see attached Lynd et al., *Microbiology and Molecular Biology Reviews*, September 2002, p. 506-577, Vol. 66, No. 3; specifically page 512, column 1, paragraph 1). It is well known in the art that the properties of enzymes can be defined by the reactions they perform and not necessarily by structural features (see attached Linton, et al., *The Journal of Experimental Biology* 207, 4095-4104, 2004) as may be the case of genetic material

outlined in *Eli Lilly*, 119 F.3d at 1568, 43 USPQ at 1406 and referred to by the Examiner. Briefly, Table 2 and results section in Linton, et al. describe various physicochemical properties such as activity, pH sensitivity, kinetics and effects of inhibition of cellulases from *G. natalis* and *D. hitipes*. It is quite common to claim an isolated enzyme or a composition with a certain enzymatic activity defined by its origin, enzymatic properties and the physicochemical characteristics such as activity, substrate specificity, working pH and temperature ranges, effects of chelating agents and proteases. For example, see Aoki, et al. (U.S. Patent No. 6,350,601) and Kimoto, et al. (U.S. Patent No. 6,969,600). Both patents disclose a reduction to practice described by physicochemical characteristics and not structural features (see, '601 patent col 7, line 22; and '600 patent col 6, line 51), even though they were issued after *Eli Lilly*, 119 F.3d at 1568, 43 USPQ at 1406.

With respect to the cellulase claimed herein, the cellulase performs a specific chemical reaction, *i.e.*, a hydrolysis of cellulose at β-1,4-glucan linkages as described in paragraph [0005] of the instant specification. In addition, the present application recites various chemical and physicochemical properties such as specificity and activity (Examples 5-8 and 12, Pages 26-38 and 54-68), effects of temperature (Examples 7-8, Pages 31-38), pH (Examples 7-8, Pages 31-38), chelating agents (Example 10C, Page 51) and proteases (Example 10B, Page 50). Thus, one skilled in the art could easily predict and/or recognize the identity of the members of the cellulase family by employing fungus from the *Chrysosporium* genus and isolating a composition with cellulase activity as described in the claimed invention. For the reasons outlined above, applicants assert that the subject of the instant application is fully enabled and

complies with the written description requirement. Reconsideration and withdrawal of the written description rejection under 35 U.S.C. §112, first paragraph is respectfully requested.

With respect to the Examiner's contention that the specification does not describe and define any structural features and amino acid sequences, while referring to applicants' acknowledgement that the structures of the cellulases were not known at the time of filing is inappropriate. Based on such an argument, the applicants should have delayed filing until additional information was obtained to further characterize their invention even though they have enably disclosed and claimed, in the same terms as originally disclosed, inventions that advanced the art.

The Examiner is well aware that the purpose of the written description is to establish that the inventor was in possession of the invention at the time the application was filed. Claims 2-66 are directed to compositions obtained from a wild type or mutant fungus. Support for these compositions as described in the previous response to the Office Action may be found in the instant specification as published at paragraphs [0026] - [0028] and [0051] - [0052], Examples 1-17, as well as the claims as originally filed. Claims 80-83 are directed to a method for generating mutant strains of *Chrysosporium* and the mutant generated therefrom. Support may be found in the instant specification as published at paragraph [0052] and Example 14. Therefore, the instant specification clearly supports the claimed invention directed to compositions obtained from wild type or mutant *Chrysosporium* and methods of producing the compositions, as well as reasonably conveys to one skilled in the art that the applicants had possession of the claimed invention at the time of filing.

Lastly, applicants respectfully direct the Examiner's attention to the quote from 66 FR 1099, Friday, January 5, 2001 presented in the recent office action at pages 2-3 which discusses guidelines for examination of patent applications under 35 U.S.C. §112, first paragraph, written description requirement in view of *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. "*Eli Lilly* explains that a chemical compound's name does not necessarily convey a written description of the named compound, particularly when a genus of compounds is claimed." The emphasis is on the term "necessarily." The recited quote does not stipulate that a chemical compound's name is inadequate as a written description. It simply states that it might not be enough. However, there are circumstances where a name is sufficient to describe a compound of interest, such as the claimed invention by stating that the composition has cellulase activity (hydrolysis of cellulose at β-1,4-glucan linkages) unique to the cellulase family of enzymes isolated from wild-type or mutant fungus of the genus *Chrysosporium*. Therefore, applicants respectfully point out to the Examiner that the family of cellulase enzymes isolated from the fungus of the genus *Chrysosporium* with physicochemical properties as outlined in the instant specification clearly defines the claimed invention. Thus one skilled in the art would recognize and distinguish the members of the cellulase family claimed in the instant invention.

In another relevant quote, applicants wish to direct the Examiner's attention to lines 1-30 of 66 FR 1099, Friday, January 5, 2001 on page 1101, 2nd column, which states that a:

> "[d]escription of an actual reduction to practice offers an important "safe haven" that applies to all applications and is just one of several ways by which an applicant may

> demonstrate possession of the claimed invention. Actual reduction to practice may be crucial in the relatively rare instances where the level of knowledge and level of skill are such that those of skill in the art cannot describe a composition structurally, or specify a process of making a composition by naming components and combining steps, in such a way as to distinguish the composition with particularity from all others. Thus, the emphasis on actual reduction to practice is appropriate in those cases where the inventor cannot provide an adequate description of what the composition is, and a definition by function is insufficient to define a composition ``because it is only an indication of what the [composition] does, rather than what it is.'' Eli Lilly, 119 F.3d at 1568, 43 USPQ at 1406. See also Amgen Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991)." (emphasis added)

Thus, applicants wish to emphasize that the instant application presents a description of an actual reduction to practice (see *Brunswick Corp. v. U.S.*, 34 Fed. Cl. 532, 584, 1995 for definition) and clearly demonstrates possession of the claimed invention. Support for this assertion may be found throughout the instant specification, in particular Examples 1-17. Specifically, Examples 1-3 of the instant specification describe how the strain of *Chrysosporium lucknowense* was isolated and characterized. Examples 4 and 5 demonstrate how a composition with cellulase activity of *Chrysosporium lucknowense* was prepared and tested. Example 8 demonstrates how compositions with cellulase activity of *Chrysosporium lucknowense* (ATCC-44006), *Chrysosporium pannorum* (ATCC-34151), *Chrysosporium pruinosum* (ATCC-24782), *Chrysosporium keratinophilum* (VKMF-2119 and VKMF-2875), *Chrysosporium lobatum* (VKMF-2120), *Chrysosporium merdarium* (VKMF-2121), *Chrysosporium queenslandicum* (VKMF-2116 and VKMF-2117) and *Chrysosporium tropicum* (VKMF-2877) genera were isolated and tested. Therefore, such extensive actual reduction to practice clearly demonstrates that the applicant had the possession of the claimed

invention and for that reason satisfies the written description requirement under under 35 U.S.C. §112, first paragraph.

For the above reasons, reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph are respectfully requested.

CONCLUSION

Based on the foregoing remarks, the applicants respectfully request reconsideration and withdrawal of the pending rejections and allowance of this application. The applicants respectfully submit that the instant application is in condition for allowance. Entry of the response and an action passing this case to issue is therefore respectfully requested. In the event that a telephone conference would facilitate examination of this application in any way, the Examiner is invited to contact the undersigned at the number provided. Favorable action by the Examiner is earnestly solicited.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. **13-4500**, Order No. 3123-4000US2.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **13-4500**, Order No. 3123-4000US2.

Respectfully submitted,
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Gene, 81 (1989) 83–95
Elsevier

GENE 03105

Cellulase families revealed by hydrophobic cluster analysis

(Computer analysis; primary structure; sequence comparison; homology; active site; endoglucanases; cellobiohydrolases; xylanases)

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Received by J.K.C. Knowles: 3 February 1989
Revised: 28 March 1989
Accepted: 29 March 1989

SUMMARY

The amino acid sequences of 21 β-glycanases have been compared by hydrophobic cluster analysis. Six families of cellulases have been identified on the basis of primary structure homology: (*A*) endoglucanases B, C and E of *Clostridium thermocellum*; endoglucanases of *Erwinia chrysanthemi* and *Bacillus sp.*; endoglucanase III of *Trichoderma reesei*; endoglucanase I of *Schizophyllum commune*; (*B*) cellobiohydrolase II of *T. reesei*; endoglucanases of *Cellulomonas fimi* and *Streptomyces sp*; (*C*) cellobiohydrolases I of *T. reesei* and of *Phanerochaete chrysosporium*; endoglucanase I of *T. reesei*; (*D*) endoglucanase A of *C. thermocellum* and an endoglucanase from *Ce. uda*; (*E*) endoglucanase D of *C. thermocellum* and an endoglucanase from *Pseudomonas fluorescens*; (*F*) xylanases of *C. thermocellum* and of *Cryptococcus albidus* and the cellobiohydrolase of *Ce. fimi*. For each family, conserved potentially catalytic residues have been listed and previous allocations of the active-site residues are evaluated in the light of the alignment of the amino acid sequences. A strong homology is also reported for the putative cellulose-binding domains of cellulases of *Ce. fimi* and of *P. fluorescens*.

INTRODUCTION

Cellulolytic fungi and bacteria produce a spectrum of cellulases which are commonly classified into two major components: 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4, endoglucanase, hereafter EG) and 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91, cellobiohydrolase, hereafter CBH). These enzymes have

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Abbreviations: aa, amino acid(s); *B.*, *Bacillus*; *C.*; *Clostridium*; CBH, cellobiohydrolase (exoglucanase); *Ce.*, *Cellulomonas*; 2D, two-dimensional; 3D, three-dimensional; *E.*, *Erwinia*; EG, endoglucanase; HCA, hydrophobic cluster analysis (Gaboriaud et al., 1987); *P.*, *Pseudomonas*; *T.*, *Trichoderma*; Xyn, xylanase.

been found to show different types of synergy when hydrolyzing crystalline cellulose (Henrissat et al., 1985). Structure-function relationships have been established in two CBHs from the fungus *Trichoderma reesei*: a conserved domain (at the C-terminus of CBH I and the N-terminus of CBH II) has been shown to be important for the activity of these enzymes on native cellulose (Tomme et al., 1988). These domains have been identified as the tails in the 'tadpole' structures which are typical of these enzymes (Teeri et al., 1987; Abuja et al., 1988). The presence of such terminal domains in cellulases from different origins is now well documented (Joliff et al., 1986; Warren et al., 1986; Knowles et al., 1987) and suggests that these functionally related enzymes might have a similar architecture. The active-site regions, however, have been postulated to be located outside these terminal domains. This has been experimentally proven for CBH I and CBH II from *T. reesei* (Tomme et al., 1988) and the enzymes from *Cellulomonas (Ce.) fimi* (Miller et al., 1988).

A number of cellulase genes have been cloned and more than 20 have been sequenced. Comparisons of cellulase sequences by classical methods have only detected homologies between cellulases from the same organism (Knowles et al., 1987) and in some cases, from two different fungi (Saloheimo et al., 1988) or different bacteria (Giuseppi et al., 1988; Horikoshi and Fukumori, 1988). This could either mean that for enzymes of widely different origin, there are no interspecies homologies, or that they have not been detected.

Hydrophobic cluster analysis (HCA) has recently proven to be a powerful method for comparing aa sequences (Gaboriaud et al., 1987; Benchetrit et al., 1988; Henrissat et al., 1988). This approach can clearly detect similarities in the 3D folding of proteins of very low sequence identity (e.g., 10%). HCA is also very effective in finding homologous domains which are separated by variable segments of widely differing sizes (Henrissat et al., 1988). In addition, HCA also brings accurate secondary structure predictions for numerous β-strands and α-helices present in globular proteins (Gaboriaud et al., 1987).

HCA has been applied to 21 available aa sequences of β-glycanases from various origins. We report here the first evidence for a series of homologies between fungal and bacterial cellulases and the finding that all the sequences available could be classified into six families of homologous proteins. Because active and/or substrate-binding sites are often highly conserved in homologous domains (Chothia and Lesk, 1986), potentially catalytic aa have been searched in an attempt to determine the essential regions for activity.

EXPERIMENTAL AND DISCUSSION

(a) Hydrophobic cluster analysis (HCA)

The major principles of the sequence representation (HCA plot) used for HCA will be recalled here for an easier understanding of the Figs. and Tables. This representation is derived from a 2D pattern already used by Lim (1974) for secondary structure predictions. The aa sequence, in one-letter code, is drawn on a classical α-helical net (3.6 residues per turn) and duplicated (Gaboriaud et al., 1987). All the sequences used in this work were from published literature (Table I). Proline is symbolized by ★, glycine by ◆, cysteine by ©, serine by ⊡ and threonine by □. The following aa are considered as hydrophobic: V, I, L, F, W, M, Y. In a hydrophobic environment, alanines and cysteines can sometimes be considered as hydrophobic. Clusters composed of adjacent hydrophobic residues not separated by prolines, are circled.

The comparisons are based on the 2D topology and distribution of the hydrophobic clusters along the sequences. Nearly all of these clusters correspond to regular secondary structure elements (Gaboriaud et al., 1987), which constitute the main 3D folding cores of globular proteins. Therefore, the comparisons are not based essentially on the maximization of sequence identities, but on the successive correspondence of 2D structuring elements (hydrophobic clusters). An alignment of aa sequences is satisfactory when correspondence of successive clusters is respected. When hydrophilic segments situated between clusters are not conserved or differ in their length, we consider that they correspond to loops and that they do not dramatically influence protein folding.

A particularly useful feature of the method is that,

TABLE I

Classification of cellulases into families of homologous proteins

Family	Code	Enzyme	Source	Reference
A	A_1	EG	*Bacillus subtilis*	Nakamura et al. (1987)
	A_2	EG C	*Clostridium thermocellum*	Schwarz et al. (1988)
	A_3	EG III	*Trichoderma reesei*	Saloheimo et al. (1988)
	A_4	EG E	*Clostridium thermocellum*	Hall et al. (1988)
	A_5	EG B	*Clostridium thermocellum*	Grépinet and Béguin (1986)
	A_6	EG	*Bacillus sp.* strain 1139	Fukumori et al. (1986a)
	A_7, A_8	EG	*Bacillus sp.* strain N-4 (genes pNK1 and pNK2)	Fukumori et al. (1986b)
	A_9	EG Z	*Erwinia chrysanthemi*	Giuseppi et al. (1988)
	A_{10}	EG I	*Schizophyllum commune*	Saloheimo et al. (1988)
B	B_1	CBH II	*Trichoderma reesei*	Chen et al. (1987); Teeri et al. (1987)
	B_2	EG A	*Cellulomonas fimi*	Wong et al. (1986)
	B_3	EG	*Streptomyces sp.* (KSM-9)	Nakai et al. (1988)
C	C_1	CBH I	*Phanerochaete chrysosporium*	Sims et al. (1988)
	C_2	CBH I	*Trichoderma reesei*	Shoemaker et al. (1983)
	C_3	EG I	*Trichoderma reesei*	Penttilä et al. (1986); Van Arsdell et al. (1987)
D	D_1	EG	*Cellulomonas uda*	Nakamura et al. (1986)
	D_2	EG A	*Clostridium thermocellum*	Béguin et al. (1985)
E	E_1	EG D	*Clostridium thermocellum*	Joliff et al. (1986)
	E_2	EG	*Pseudomonas fluorescens*	Hall and Gilbert (1988)
F	F_1	CBH	*Cellulomonas fimi*	O'Neill et al. (1986)
	F_2	Xyn Z	*Clostridium thermocellum*	Grépinet et al. (1988)
	F_3	Xyn	*Cryptococcus albidus*[a]	Morolosi and Durand (1988)

[a] Partial sequence.

given the compactness of HCA sequence representation, the simultaneous comparison of up to 30–40 proteins is possible. Within a family of distantly related proteins the distribution of clear filiations vary from one segment to another and only such a simultaneous comparison allows a consistent alignment. Moreover, it shows evolutionary filiations between clusters and this cannot be easily detected otherwise. The study of family A is a direct application of such a multi-sequence handling.

Homology was estimated within the homologous domains as the percentage of match after linear alignment of the sequences according to the indications of HCA. In addition, HCA homology scores were calculated (Gaboriaud et al., 1987). If a fair HCA score (more than 60%) is obtained along wide segments of their sequences, the method can detect 3D homologous proteins even if their sequence identity is as low as 10% (Gaboriaud et al., 1987).

(b) Family A

Figure 1 features the HCA plots of selected bacterial (*Bacillus subtilis*; *C. thermocellum*) and fungal (*T. reesei*) EGs of family A (Table I). A number of hydrophobic clusters, spanning more than 150 aa, have similar shapes, sizes and orientations throughout their aa sequences. The occurrence of these conserved segments (numbered S_1 to S_5), alternating with more variable ones (V_1 to V_3), clearly

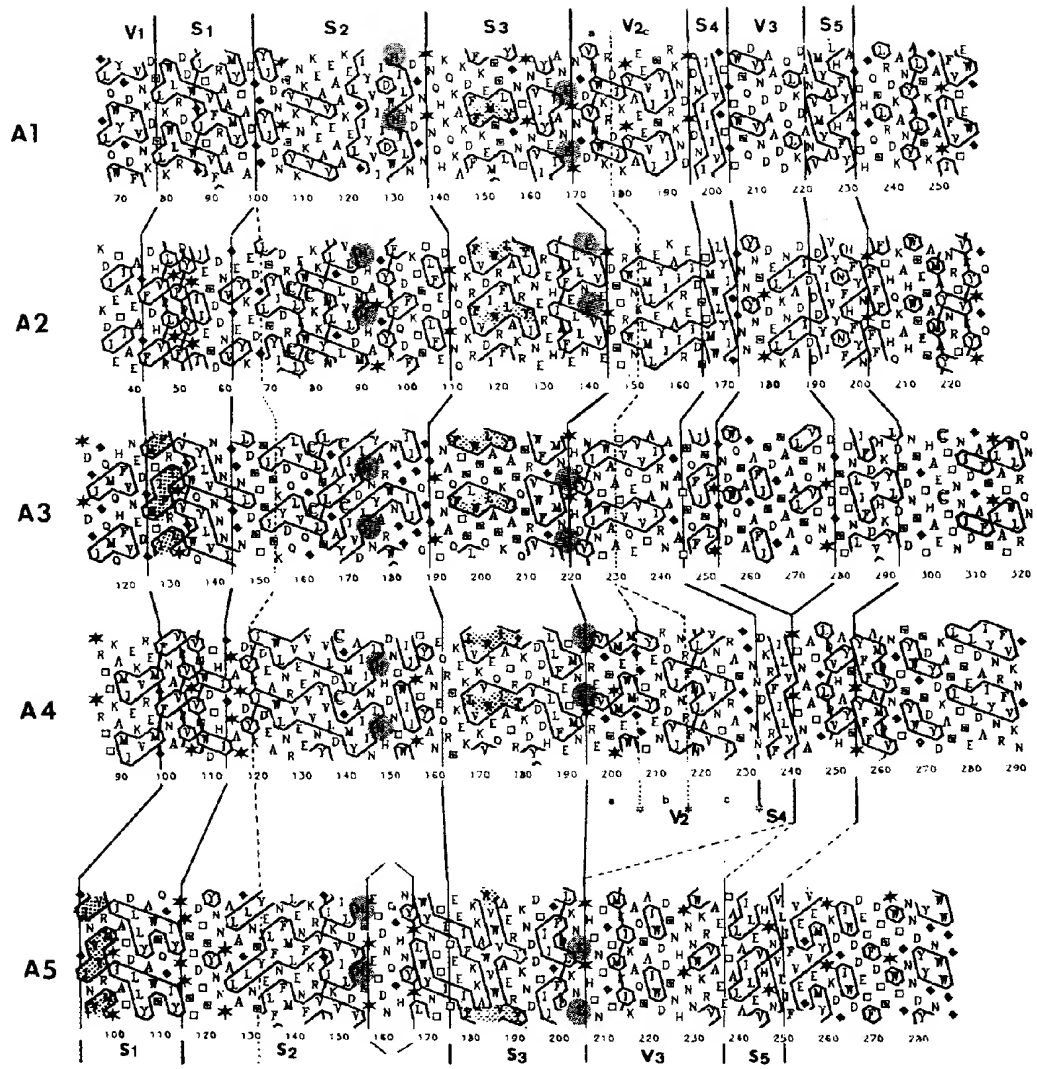

Fig. 1. HCA plots of the conserved domain in selected cellulases from family A (A_1–A_5; see Table I). Well-conserved segments (S) and more variable ones (V) are shown. Vertical lines indicate the proposed correspondences between the segments. Proline is symbolized by , glycine by ◆, cysteine by ©, serine by ⊡ and threonine by □. Conserved His and Glu residues are shaded. Conserved residue patterns used for alignment are stippled. Clusters of hydrophobic aa are boxed. Homology scores are reported in Table II.

demonstrates the homology of these proteins. No conserved cysteine residue could be traced in this family. The homology scores within the conserved region are reported in Table II. The similarity between EG B of *C. thermocellum* and the other enzymes of this family was slightly more difficult to detect because it is apparent only in segments S_1, S_2 and S_3. For the rest of the sequence of EG B, the alignment is only suggested (Fig. 1).

EGs from *E. chrysanthemi* and from alkalophilic *Bacillus sp.*, which have been previously found to be homologous to *B. subtilis* EG (Giuseppi et al., 1988) are also members of this family (HCA plots not shown). It is worth mentioning that this family probably also includes *Schizophyllum commune* EG I, whose complete primary structure is not yet available, but whose homology with EG III of *T. reesei* has been reported (Saloheimo et al., 1988).

(c) Family B

The HCA plots of EG A of *Ce. fimi*, CBH II of *T. reesei* and EG of *Streptomyces sp.* strain KSM-9 are presented in Fig. 2. A previously unreported

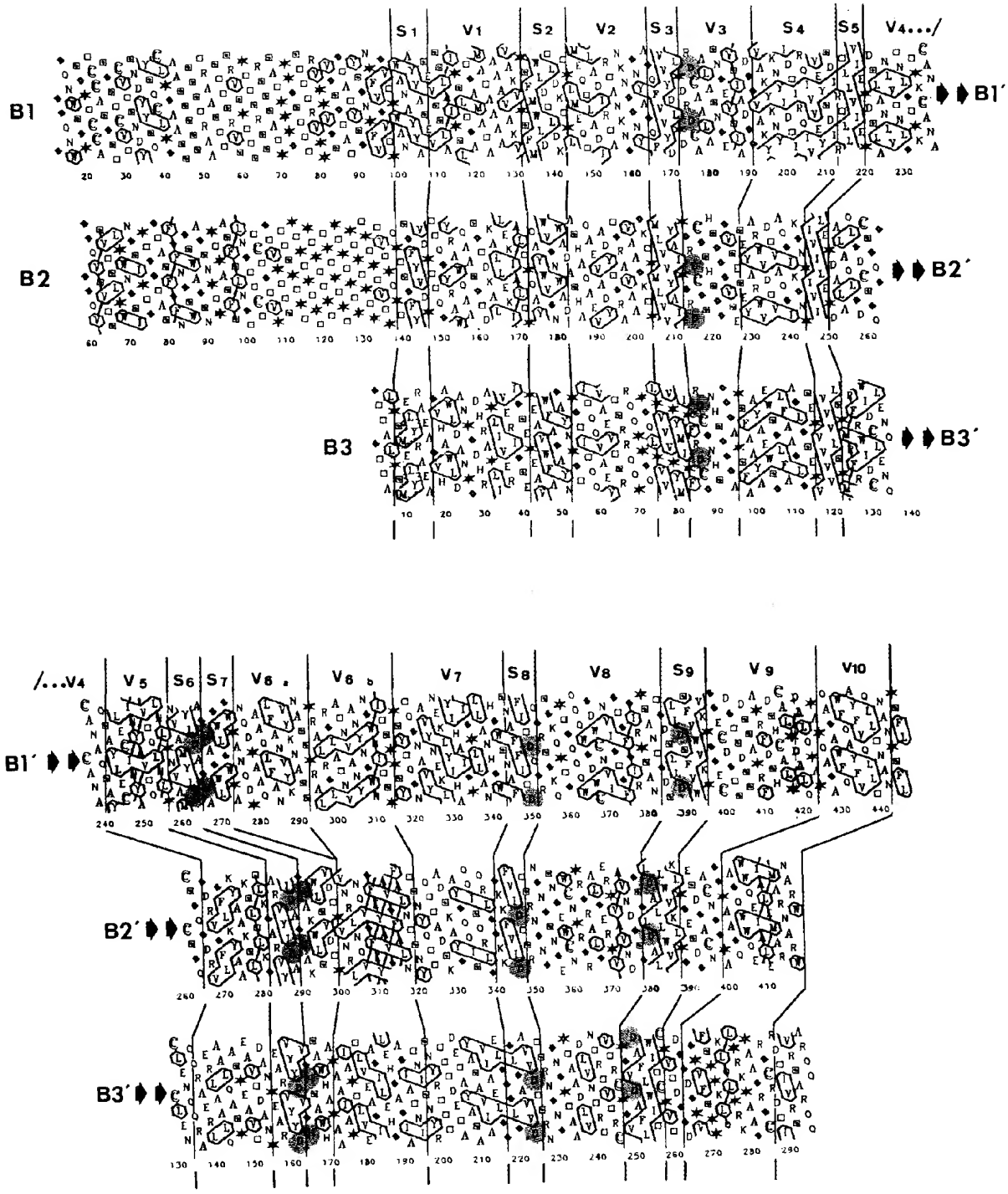

Fig. 2. HCA plots of cellulases from family B. B_1 = CBH II of *T. reesei*, B_2 = EG A of *Ce. fimi* and B_3 is the endoglucanase of *Streptomyces sp.* strain KSM-9. B_1', B_2' and B_3' are the continuation of B_1, B_2 and B_3. Well-conserved segments (S) and more variable ones (V) are shown. Vertical lines indicate the proposed correspondences between segments. Conserved His and Asp residues are shaded. HCA scores: B_1/B_2, 77%; B_2/B_3, 67%; B_1/B_3, 69%.

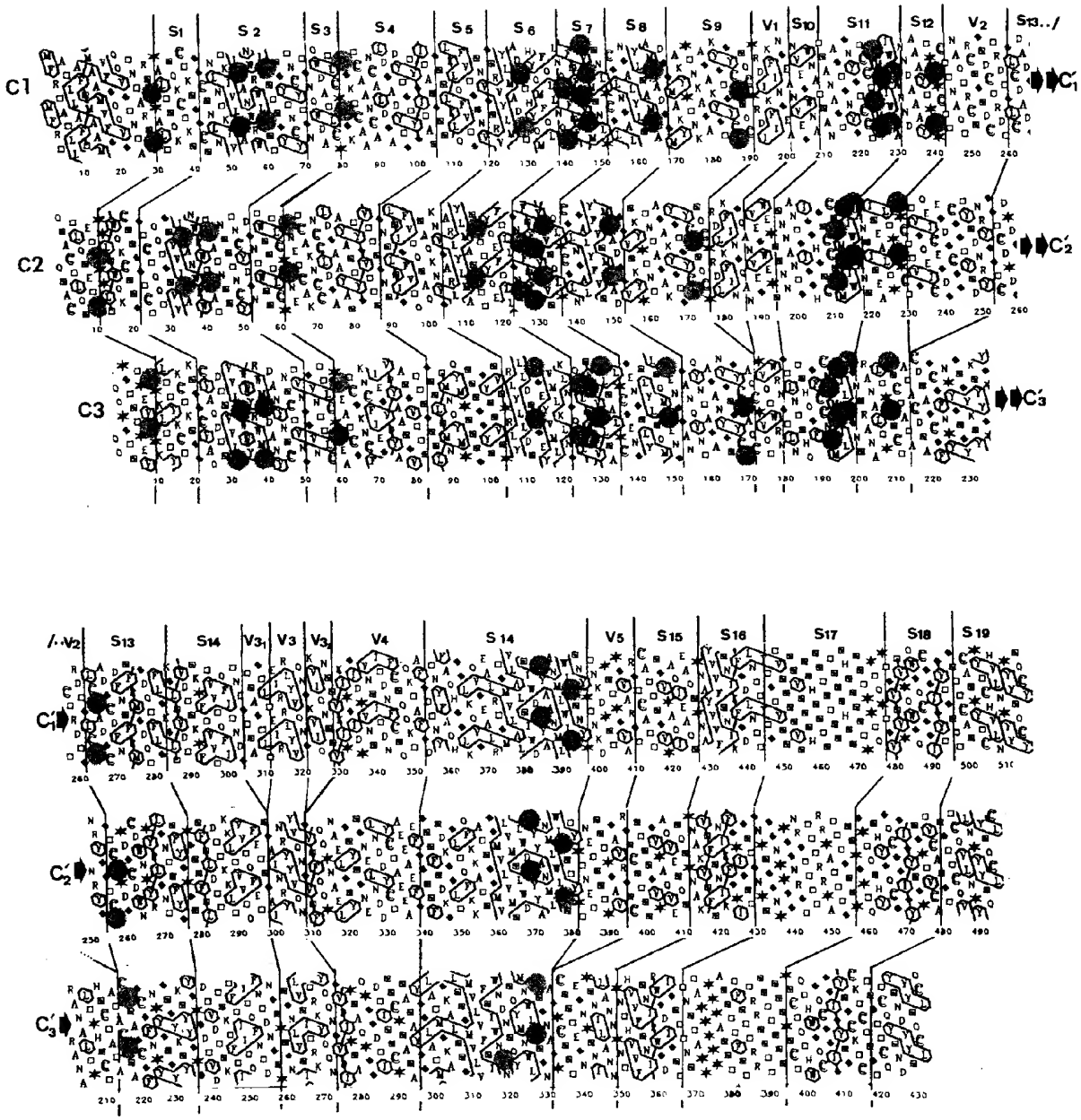

Fig. 3. HCA plots of cellulases from family C. C_1 = CBH I of *Phanerochaete chrysosporium*, C_2 = CBH I of *T. reesei* and C_3 = EG I of *T. reesei*. C_1', C_2' and C_3' are the continuation of C_1, C_2 and C_3. Vertical lines indicate the proposed correspondences between the sequences. Conserved His, Glu and Asp residues are shaded. HCA scores: C_1/C_2, 89%; C_2/C_3, 77%; C_1/C_3, 76%.

homology between these bacterial and fungal proteins becomes evident here since a series of similar hydrophobic cluster can be detected over more than 250 aa. Within its 'core', EG A displays 20 and 22% aa identity, respectively, with CBH II and the EG of *Streptomyces*. The homologous segments (numbered S_1 to S_9), which alternate with more variable ones (numbered V_1 to V_{10}), probably represent the 'building blocks' constituting the core of this family of proteins. Two cysteine residues (176 and 235 in CBH II) are conserved at equivalent positions. Between segments, many probable loops could be delineated, e.g., GGV around 190 in CBH II, 223 in EG A and GGA around 93 in *Streptomyces* EG.

TABLE II

Homology scores in the conserved domain of cellulases from family A[a,b]

Codes[c]	A_1	A_2	A_3	A_4	A_5
A_1	100 (100)				
A_2	15 (66)	100 (100)			
A_3	17 (74)	15 (67)	100 (100)		
A_4	14 (69)	15 (66)	19 (69)	100 (100)	
A_5	12 (64)	11 (65)	11 (65)	14 (64)	100 (100)

[a] Enzymes (A_6–A_9) have been omitted since their homology with (A_1) has been already reported (Giuseppi et al., 1988).

[b] The first score is the aa identity in the conserved domain after alignment according to HCA. The score in parentheses is the HCA score within the conserved domain (Gaboriaud et al., 1987).

[c] Enzyme codes are defined in Table I.

(d) Family C

The HCA plots of CBH I and EG I of *T. reesei* are displayed in Fig. 3. The homology between the two sequences is obvious and confirms earlier findings (Bhikhabhai and Pettersson, 1984; Penttilä et al., 1986; Van Arsdell et al., 1987). Alignment of the homologous segments (numbered S_1 to S_{19}) found throughout both sequences reveals that there are regions of CBH I (e.g. 177–188; 230–253 and 382–395) which are absent in EG I. Eighteen cysteine residues are conserved in both enzymes and the overall aa identity is about 48% (Van Arsdell et al., 1987). CBH I of *Phanerochaete chrysosporium* (Sims et al., 1988) was found to be highly similar to CBH I of *T. reesei* (approx. 70% residue identity) and, therefore, can be classified in this family.

(e) Family D

This fourth family of cellulases features two bacterial cellulases, namely EG A of *C. thermocellum* and an EG of *Ce. uda*, whose HCA plots are reported in Fig. 4. Similar segments are numbered S_1 to S_{10}. There is no cysteine residue conserved in the two sequences. The N-terminal half of these two enzymes is better conserved than their C-terminal half and the aa identity within the conserved region (200 aa from S_1 to S_{10}) is approx. 24%. The Pro + Thr-rich region of EG A matches the C-terminus of the *Ce. uda* enzyme.

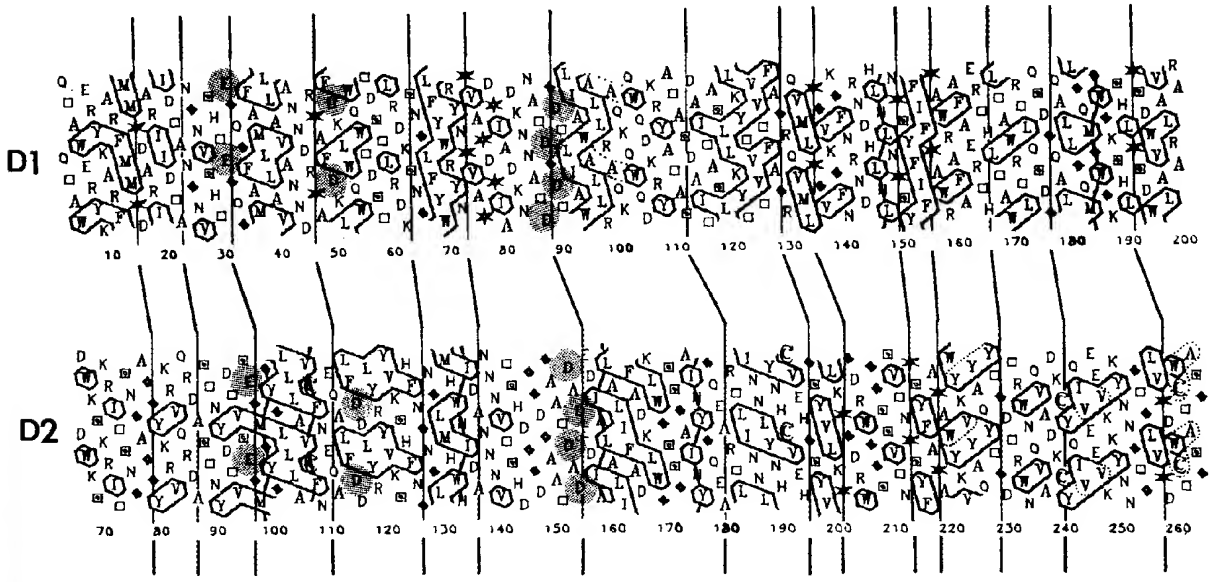

Fig. 4. HCA plots of cellulases from family D. D_1 = endoglucanase of *Ce. uda*. D_2 = EG A of *C. thermocellum*. Hydrophilic Tyr and hydrophobic Ala and Cys residues are circled with dotted lines to emphasize homologies between clusters. Vertical lines indicate the proposed correspondences between the two sequences. Conserved Glu and Asp residues are shaded. HCA score: 70%.

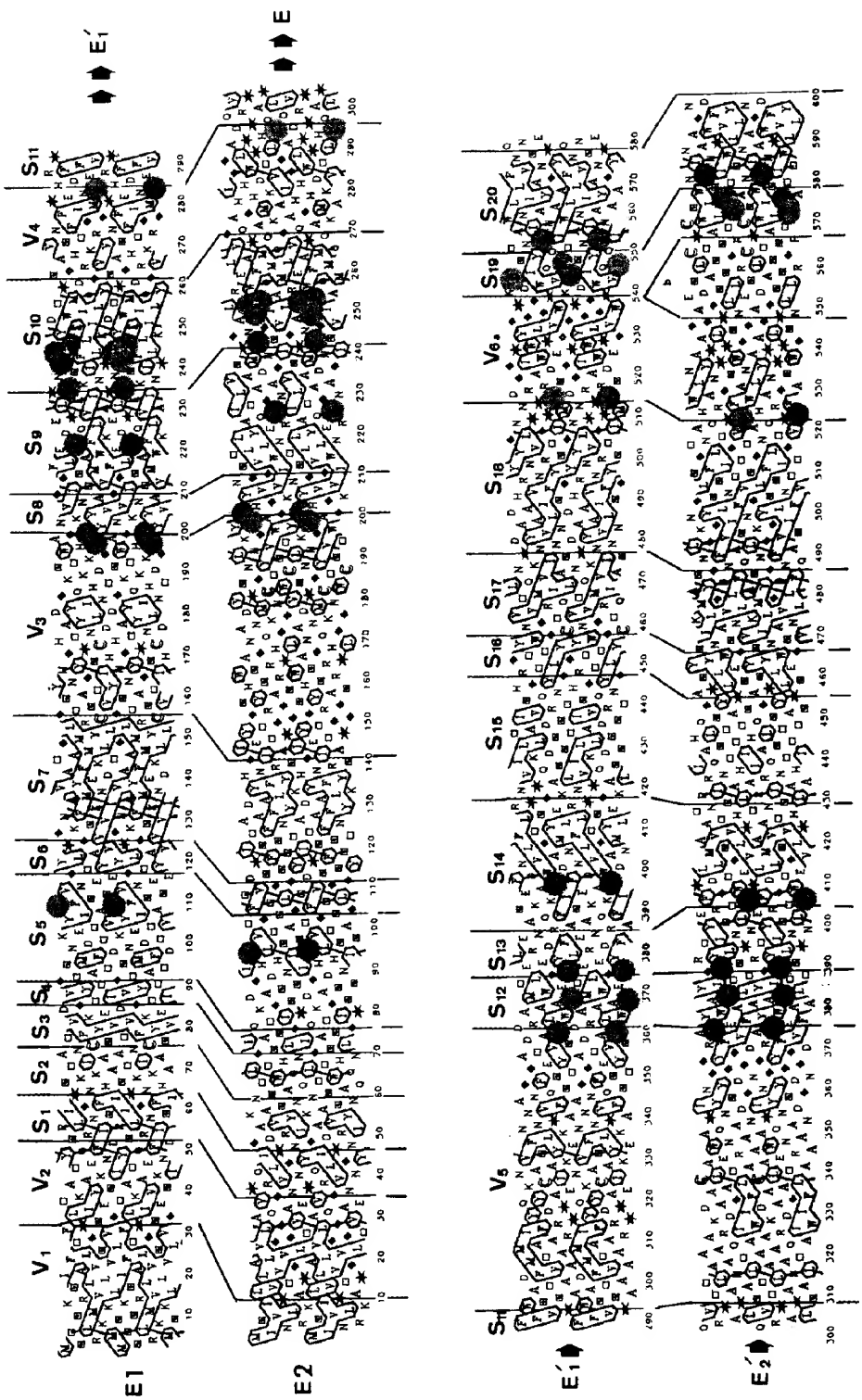

Fig. 5. HCA plots of cellulases from family E. E_1 = EG D of *C. thermocellum*. E_2 = endoglucanase of *P. fluorescens*. E_1' and E_2' indicate the continuation of E_1 and E_2. Well-conserved segments (S) and more variable ones (V) are shown. Vertical lines indicate the proposed correspondences between the segments. Conserved His, Asp and Glu residues are shaded. HCA score: 72% (calculated without taking into account the variable segments).

(f) Family E

The HCA plots of EG D of *C. thermocellum* and the endoglucanase of *Pseudomonas fluorescens* subsp. *cellulosa* are given in Fig. 5. A number of hydrophobic aa clusters with similar shapes, sizes and orientations are found throughout both sequences. The homology spans nearly 600 aa and corresponds roughly to the 'core' of the proteins. In the 'cores', the aa identity is about 27%. The C-terminal domains have very different sizes (approx. 70 aa for EG D and over 350 for the *Pseudomonas* enzyme) and their primary structures do no seem to be homologous. No conserved cysteine residue could be detected in these proteins.

(g) Family F

The HCA plot of CBH of *Ce. fimi* is presented in Fig. 6 along with the plot of xylanase (Xyn) Z of *C. thermocellum*. The homology between these two enzymes is readily apparent, since similar hydrophobic clusters can be detected over 260 aa residues. In the homologous region the aa identity reaches approx. 40%. Two cysteines are conserved in both sequences (261, 267 in the *Cellulomonas* enzyme and 284, 290 in Xyn Z). It is interesting to note that this family is constituted of non-specific glycanases which hydrolyze 1,4-β-D-glucan and 1,4-β-D-xylan. Hydrolysis of xylan by certain cellulases has been proven in several cases (Shoemaker et al., 1983; Biely and Markovic, 1988) including CBH of *Ce. fimi* (Curry et al., 1988). A partial N-terminal sequence of a Xyn of the yeast *Cryptococcus albidus* (Morosoli and Durand, 1988) was found to be homologous to Xyn Z of *C. thermocellum* (HCA plot not shown), and it is therefore classified in the same family.

(h) The various cellulase families

The comparison of cellulase sequences showing very little homology in composition and length has become possible through the powerful capabilities of HCA. Six different families of cellulases have been identified (Table I). Except within certain putative binding domains, no inter-family homology could be detected by this method (HCA scores $<50\%$). Families A and B both contain fungal and bacterial cellulases. So far, family C contains only fungal enzymes, whereas families D–F are composed of bacterial cellulases. Apart from families C and F, which contain fairly homologous enzymes, the low homologies found within each of the other families suggest that the primary structures of cellulases have extensively diverged from those of their ancestors. Cellulases represent an interesting evolutionary scheme, since in some cases their two domains ('core' and 'binding') appear to have evolved from different ancestors. This is the case of the *P. fluorescens* EG, whose core is homologous to that of EG of *C. thermocellum* (Family E), whereas its C-terminal domain is significantly homologous to the 'binding' domain of the *Ce. fimi* enzymes (Families B and F).

Over the last 15 years many authors have attempted to classify cellulases in terms of *endo-* (EG) or *exo-* (CBH) glucanase activity. This has led to controversies which resulted from (i) the use of widely varying and sometimes poorly defined substrates and (ii) from the uncertainly of the ultimate purity of so-called *exo*-glucanases, sometimes contaminated by minute amounts of endoglucanases. The identification of different families by the method described in this work provides the opportunity for a less controversial classification of β-glycanases, based on the primary structure homology of their 'cores' (Table I). It would be interesting to compare the enzymatic properties of cellulases in light of this new classification.

(i) Possible location of the active site

It is generally accepted that enzymatic hydrolysis of glycosidic bonds proceeds through general acid catalysis, usually promoted by aspartic or glutamic acid residues. This leads to an intermediate carbonium ion, which is stabilized by a negatively-charged group (aspartate, glutamate) or by a histidine residue (Paice and Jurasek, 1979; Zvelebil and Sternberg, 1988). Since active and/or substrate binding sites are often highly conserved, we have listed the Asp, Glu and His residues which are conserved in homologous regions of the ten sequences of family A (Table III). Two such residues are conserved: His in segment S_2, and Glu at the end of segment S_3 (Fig. 1). Among the sequences in our hands, these two residues are found in well-conserved regions and

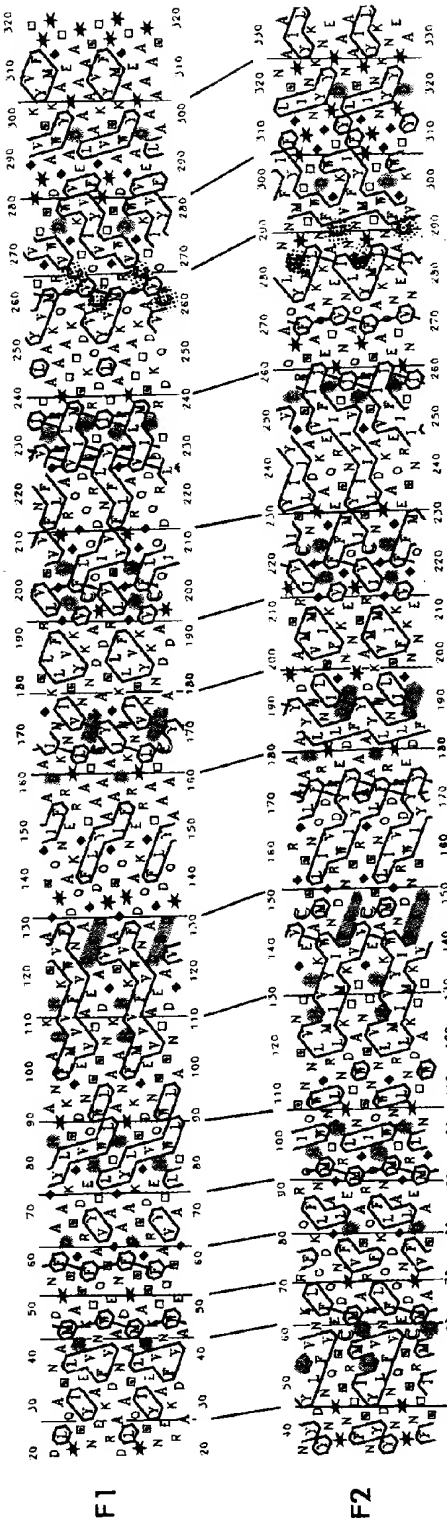

Fig. 6. HCA plots of cellulases from family F. F_1 = CBH of *Ce. fimi*. F_2 = Xyn Z of *C. thermocellum*. Vertical lines indicate the proposed correspondences between the two sequences. Conserved His, Asp and Glu residues are shaded, whereas conserved Cys residues are stippled. HCA score: 78%.

TABLE III

List of Asp (D), Glu (E) and His (H) residues conserved at equivalent topological positions in the various cellulases families[a]

Code[b]	Residue number				
A_1[c]	131 (H)	169 (E)			
A_2[c]	90 (H)	140 (E)			
A_3	174 (H)	218 (E)			
A_4[c]	148 (H)	193 (E)			
A_5[c]	155 (H)	204 (E)			
A_6	121 (H)	160 (E)			
A_7[c]	125 (H)	163 (E)			
A_8[c]	127 (H)	165 (E)			
A_9	98 (H)	133 (E)			
A_{10}	[d] (H)	[d] (E)			
B_1	175 (D)	263 (D)	266 (H)	350 (D)	389 (D)
B_2	216 (D)	287 (D)	290 (H)	346 (D)	380 (D)
B_3	85 (D)	162 (D)	165 (H)	223 (D)	249 (D)
D_1	30 (E)	49 (D)	87 (D)	89 (D)	217 (D)
D_2[c]	95 (E)	114 (D)	152 (D)	154 (D)	278 (D)

[a] Families C, E and F have been omitted since the lists of their conserved Asp, Glu and His residues are so long that they are not useful as active-site residue predictions.

[b] Enzyme codes are defined in Table I.

[c] Numbering includes signal peptide.

[d] Unpublished numbering, but these residues are conserved in A_{10} and A_8 (Saloheimo et al., 1988).

are separated by segments of comparable length, ranging from 35 to 50 aa. Another His residue seems conserved in segment S_5 (Fig. 1). However it was not selected as a possible active-site residue, because it is not conserved in *Schizophyllum commune* EG I (Saloheimo et al., 1988).

A similar analysis of the conserved catalytic residues is theoretically possible with the five other families of homologous cellulases (B-F). However, since these families contain, so far, only a few numbers each (Table I), increased uncertainty occurs with 5, 17, 5, 17 and 18 potentially catalytic residues, respectively, conserved in families B, C, D, E and F (Table III). When sequences of other members of these families become available, one should be able to further eliminate non-essential residues and localize more precisely the aa involved in catalysis. It can be mentioned that out of the two catalytic residues proposed (Chen et al., 1987) for *T. reesei* CBH II (Asp-175 and Glu-184), Glu-184 is not conserved in EG A of *Ce. fimi* (Table III), thus suggesting that this residue is not directly involved in catalysis. Similarly, the three active-site residues proposed (Warren et al., 1986) for *Ce. fimi* EG A (Glu-309, Asn-317 and Thr-324) are not present in the homologous segment of CBH II (Fig. 2). It seems therefore unlikely that these non-conserved residues are participating in catalysis.

Recent active-site modification studies of CBH I of *T. reesei* (Tomme and Claeyssens, 1989) have shown that Asp (Glu) groups in the 115–130 region are implicated and therefore, the corresponding clusters (S_6 and S_7) in the HCA plot (Fig. 3) could be functionally important. The corresponding region in EG I (Fig. 3) is very well conserved. Active site-directed modifications occur very similarly (Tomme and Claeyssens, 1989) and also striking analogies in specificity can be traced in both enzymes (Van Tilbeurgh et al., 1986).

(j) Conclusions

The application of HCA to all published cellulase gene sequences has allowed the assignment of each cellulase to one of six families. Inter-family similarities were not detected, thereby suggesting that there are different folds rather than only variants of one fold. Since the major elements of the active site of a given family are expected to be conserved, we have listed the possible catalytic residues conserved in each family. The prediction of putative active-site residues in one family was thus greatly facilitated. In two other families, this approach reduced the number of potential active-site residues to only five candidates, and this should help the design of successful site-directed mutagenesis experiments.

A modular structure with a rather invariant core decorated with variable loops is a common arrangement found in a number of protein families (Chothia and Lesk, 1986). It is tempting to suggest that such an arrangement could also be valid for each family of cellulases. When 3D structures of cellulases become available, the alignments which can be derived from the present work could constitute a useful starting point for the prediction of the tertiary structure of related enzymes.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. Béguin (Institut Pasteur) and Dr. A. Bairoch (University of Geneva) for stimulating discussions. They are also indebted to D. Landry for her help in the preparation of the manuscript.

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Vol. 81

ELSEVIER SCIENCE PUBLISHERS BV—AMSTERDAM

This journal is printed on acid-free paper.

Cited in: Biological Abstracts; Chemical Abstracts; Current Contents (Life Sciences); Excerpta Medica (EMBASE); Index Medicus; Pascal M.; Science Citation Index; Telegen

Printed in Belgium

MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2002, p. 506–577 Vol. 66, No. 3
1092-2172/02/$04.00+0 DOI: 10.1128/MMBR.66.3.506–577.2002

Microbial Cellulose Utilization: Fundamentals and Biotechnology

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INTRODUCTION

Life on Earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. The carbon cycle is closed primarily as a result of the action of cellulose-utilizing microorganisms present in soil and the guts of animals. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of carbon flux at both local and global scales. The importance of microbial cellulose utilization in natural environments is further enhanced by the status of ruminants as a major source of dietary protein. Finally, microbial cellulose utilization is also an integral component of widely used processes such as anaerobic digestion and composting.

Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (410). Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply. The central technological impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products in a single process step via a cellulolytic microorganism or consortium. Such "consolidated bioprocessing" (CBP) offers very large cost reductions if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties (405).

Notwithstanding its importance in various contexts, fundamental understanding of microbial cellulose utilization is in many respects rudimentary. This is a result of the inherent complexity of microbial cellulose utilization as well as methodological challenges associated with its study. Understanding of cellulose hydrolysis can be approached at several levels of aggregation: components of cellulase enzyme systems, unfractionated cellulase systems, pure cultures of cellulolytic microorganisms, and mixed cultures of cellulolytic microorganisms. In general, our understanding is progressively less complete at more highly aggregated levels of study. Thus, although much remains to be elucidated at the level of enzyme components and the underlying genetics of such components, understanding of cellulose hydrolysis by unfractionated cellulase systems is still less complete, understanding of hydrolysis by pure cultures is more limited yet, and hydrolysis in multispecies cultures and mixed communities is least understood of all. There is a natural tendency for science to proceed over time toward a finer level of aggregation—e.g., from pathways to enzymes to genes—and this "reductionist" approach has yielded tremendous insights with respect to the life sciences generally and cellulose hydrolysis in particular. An alternative "integrative" approach, involving the development of an understanding of aggregated systems based on an understanding of their less aggregated components, is also a valid and important focus for scientific endeavor. With respect to cellulose hydrolysis, such integration is essential for research advances to be translated into advances in technological, ecological, and agricultural domains.

The great majority of cellulose hydrolysis research to date has focused on the genetics, structure, function, and interaction of components of cellulase enzyme systems. Several recent and comprehensive reviews address this large body of work (see "Cellulase enzyme systems" below). Whereas hydrolysis of cellulosic biomass has been approached in prior reviews and the research literature primarily as an enzymatic phenomenon, this review approaches the subject primarily as a microbial phenomenon. Thus, we intend our review to embody the integrative approach described in the previous paragraph.

The goals of this review are to collect and synthesize information from the literature on microbial cellulose utilization in both natural and technological contexts, to point out key unresolved issues, and to suggest approaches by which such issues can be addressed. In seeking to consider microbial cellulose utilization from an integrative perspective, we endeavor to consider a diversity of cellulolytic organisms and enzyme systems. This effort is, however, constrained by the information available, which is much more extensive for some types of systems and some levels of consideration than for others. Both aerobic and anaerobic organisms and enzymes are considered in our discussion of fundamentals (see "Fundamentals" below) and methodological aspects (see "Methodological basis for study" below). Our treatment of quantitative aspects of microbial cellulose utilization (see "Quantitative description of cellulose hydrolysis" below) of necessity focuses primarily on aerobic organisms and their enzymes. Information on anaerobic organisms and their enzymes is included in this section as possible, but is much more limited. In considering processing of cellulosic biomass (see "Processing of cellulosic biomass—a biological perspective" below) and organism development for consolidated bioprocessing (see "Organism development for consolidated bioprocessing" below), we focus on organisms producing reduced metabolic products via an effectively anaerobic metabolism because this is responsive to the needs, constraints, and opportunities associated with microbial conversion of cellulosic feedstocks (see"Processing of cellulosic biomass—a biological perspective" below). Literature pertaining to noncellulolytic organisms is included in cases where it provides important foundational understanding for topics involving cellulolytic organisms, as in the case of metabolic engineering of end product formation in cellulolytic anaerobes and expression of heterologous saccharolytic enzymes in noncellulolytic hosts (see "Organism development for consolidated bioprocessing" below). We conclude with a discussion of the genesis, status, and future direction of the microbial cellulose utilization field from both fundamental and biotechnological perspectives.

FUNDAMENTALS

Structure and Composition of Cellulosic Biomass

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates) and a few bacteria. Despite great differences in composition and in the anatomical structure of cell walls across plant taxa, a high

cellulose content—typically in the range of approximately 35 to 50% of plant dry weight—is a unifying feature (410). In a few cases (notably cotton bolls), cellulose is present in a nearly pure state. In most cases, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30% of plant dry weight (410, 428, 707). Although these matrix interactions vary with plant cell type and with maturity (748), they are a dominant structural feature limiting the rate and extent of utilization of whole, untreated biomass materials. A detailed description of these interactions and the mechanisms by which they limit hydrolysis and utilization is beyond the scope of this paper and is the topic of several recent reviews (245, 749). The discussion below is focused primarily on cellulose itself, since it appears that—once stripped of the protective effects of other plant biopolymers—cellulose in native plant material shares many characteristics across plant taxa, including its potential for complete hydrolysis and utilization under the proper microbial and environmental conditions.

An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure. Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis (86). There is evidence that associated hemicelluloses regulate this aggregation process (19). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibers.

The arrangement of individual chains within the elementary fibrils has largely been inferred from the fitting of X-ray diffraction data to statistical models that calculate structure based on minimum conformational energy. Individual models are a source of considerable controversy, even in terms of such fundamentals as the orientation of adjacent chains (parallel up versus parallel down) (354, 355, 510). Regardless of their orientation, the chains are stiffened by both intrachain and interchain hydrogen bonds. Adjacent sheets overlie one another and are held together (in cellulose I, the most abundant form of cellulose in nature) by weak intersheet van der Waals forces; despite the weakness of these interactions, their total effect over the many residues in the elementary fibril is considerable (538). The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another. An important feature of the crystalline array is that the component molecules of individual microfibrils are packed sufficiently tightly to prevent penetration not only by enzymes but even by small molecules such as water.

Although cellulose forms a distinct crystalline structure, cellulose fibers in nature are not purely crystalline. The degree of departure from crystallinity is variable and has led to the notion of a "lateral order distribution" of crystallinity, which portrays a population of cellulose fibers in statistical terms as a continuum from purely crystalline to purely amorphous, with all degrees of order in between (427). In addition to the crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks or twists of the microfibrils, or voids such as surface micropores, large pits, and capillaries (63, 127, 178, 428). The total surface area of a cellulose fiber is thus much greater than the surface area of an ideally smooth fiber of the same dimension. The net effect of structural heterogeneity within the fiber is that the fibers are at least partially hydrated by water when immersed in aqueous media, and some micropores and capillaries are sufficiently spacious to permit penetration by relatively large molecules—including, in some cases, cellulolytic enzymes (647, 648).

Purified celluloses used for studies of hydrolysis and microbial utilization vary considerably in fine structural features, and the choice of substrate for such studies undoubtedly affects the results obtained. Holocelluloses such as Solka Floc are produced by delignification of wood or other biomass materials. These materials contain substantial amounts of various hemicelluloses and often have a low bulk density suggestive of some swelling of cellulose fibers. Microcrystalline celluloses (e.g., Avicel and Sigmacell) are nearly pure cellulose, and the dilute-acid treatment used in their preparation removes both hemicelluloses and the more extensive amorphous regions of the cellulose fibers. Commercial microcrystalline celluloses differ primarily in particle size distribution, which (as indicated below) has significant implications for the rate of hydrolysis and utilization. Cellulose synthesized by the aerobic bacterium *Acetobacter xylinum* has been tremendously useful as a model system for studying cellulose biosynthesis, but has only been used for a few studies of microbial cellulose utilization. Like plant cellulose, bacterial cellulose is highly crystalline, but the two celluloses differ in the arrangement of glucosyl units within the unit cells of the crystallites (20), and genetic evidence suggests that the two celluloses are synthesized by enzymatic machinery that differs considerably at the molecular level (86). The two celluloses also differ substantially in rate of hydrolysis by fungal cellulases (246) and in rate of utilization by mixed ruminal bacteria (602, 731). The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates has led to the wide use of the highly soluble cellulose ether, carboxymethylcellulose (CMC), as a substrate for studies of endoglucanase production. Unfortunately, the use of CMC as an enzymatic substrate has weakened the meaning of the term "cellulolytic," since many organisms that cannot degrade cellulose can hydrolyze CMC via mixed β-glucan enzymes (185). Because of the substituted nature of the hydrolytic products, relatively few microbes (including some fungi and *Cellulomonas* strains) can use CMC as a growth substrate.

Utilization of cellulosic biomass is more complex than is that of pure cellulose, not only because of the former's complex composition (i.e., presence of hemicelluloses and lignin) but also because of the diverse architecture of plant cells themselves. Plant tissues differ tremendously with respect to size and organization. Some plant cell types (e.g., mesophyll) have thin, poorly lignified walls that are easily degraded by polysaccharide-hydrolyzing enzymes. Others, like sclerenchyma, have thick cell walls and a highly lignified middle lamella separating cells from one another. These cell walls must be attacked from the inside (luminal) surface out through the secondary wall (as opposed to particles of pure cellulose, which are degraded from the outside inward). Thus, in addition to constraints imposed by the structure of cellulose itself, additional limitations are imposed by diffusion and transport of the cellulolytic agent to the site of attack. These constraints may severely limit utilization in some habitats (750).

Taxonomic Diversity

Until recently, hydrolysis and utilization of cellulose in amounts sufficient to provide usable energy to an organism were thought to be carried out exclusively by microorganisms. It now appears that some animal species, including termites and crayfish, produce their own cellulases, which differ substantially from those of their indigenous microflora (723), although the contribution of these enzymes to the nutrition of the animal is unclear. In examining the distribution of cellulolytic species across taxonomic groups, it is useful to consider microbial taxonomy based on phylogeny, rather than on a set of arbitrary morphological or biochemical characteristics as used in classical taxonomy. Current views of the evolutionary relatedness of organisms are based largely on phylogenetic trees constructed from measurements of sequence divergence among chronometric macromolecules, particularly small-subunit rRNAs (16S rRNA of procaryotes and 18S rRNA of eucaryotes [503, 752]). An inspection of these trees reveals that the ability to digest cellulose is widely distributed among many genera in the domain *Bacteria* and in the fungal groups within the domain *Eucarya*, although no cellulolytic members of domain *Archaea* have yet been identified. Within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*). Fungal cellulose utilization is distributed across the entire kingdom, from the primitive, protist-like Chytridomycetes to the advanced Basidiomycetes.

The broad distribution of cellulolytic capability could suggest conservation of a cellulose-degrading capability acquired by a primordial ancestor early in evolutionary development; however, this would seem unlikely, given that the capacity for cellulose biosynthesis did not evolve until much later, with the development of algae, land plants and the bacterium *A. xylinum*. More likely is the convergent evolution toward a cellulolytic capability under the selective pressure of abundant cellulose availability following the development of cellulose biosynthesis. Evidence for such convergent evolution is discussed below (see "Molecular biology of cellulase enzymes").

Fungi are well-known agents of decomposition of organic matter in general and of cellulosic substrates in particular (94, 462). Fungal taxonomy is based largely on the morphology of mycelia and reproductive structures during various stages of the fungal life cycle rather than on substrate utilization capability. Indeed, systematic characterization of growth substrates has not been carried out for many described fungal species. Therefore, it is currently unclear how broadly and deeply cellulolytic capability extends through the fungal world, and a consideration of the taxonomy of cellulolytic fungi may ultimately prove to be only a slightly narrower topic than consideration of fungal taxonomy in its entirety. Nevertheless, some generalizations can be made regarding the distribution of cellulolytic capabilities among these organisms.

A number of species of the most primitive group of fungi, the anaerobic Chytridomycetes, are well known for their ability to degrade cellulose in gastrointestinal tracts of ruminant animals. Although taxonomy of this group remains controversial (94), members of the order Neocallimastigales have been classified based on the morphology of their motile zoospores and vegetative thalli; they include the monocentric genera *Neocallimastix, Piromyces*, and *Caecomyces* and the polycentric genera *Orpimomyces* and *Anaeromyces* (376). Cellulolytic capability is also well represented among the remaining subdivisions of aerobic fungi. Within the approximately 700 species of Zygomycetes, only certain members of the genus *Mucor* have been shown to possess significant cellulolytic activity, although members of this genus are better known for their ability to utilize soluble substrates. By contrast, the much more diverse subdivisions Ascomycetes, Basidiomycetes, and Deuteromycetes (each of which number over 15,000 species [94]), contain large numbers of cellulolytic species. Members of genera that have received considerable study with respect to their cellulolytic enzymes and/or wood-degrading capability include *Bulgaria, Chaetomium*, and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); and *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium*, and *Trichoderma* (Deuteromycetes). For a more detailed consideration of fungal taxonomy and some of its unresolved issues, see reference 94.

When viewed through the lens of microbial physiology, the cellulolytic bacteria can be observed to comprise several diverse physiological groups (Table 1): (i) fermentative anaerobes, typically gram positive (*Clostridium, Ruminococcus*, and *Caldicellulosiruptor*) but containing a few gram-negative species, most of which are phylogenetically related to the *Clostridium* assemblage (*Butyrivibrio* and *Acetivibrio*) but some of which are not (*Fibrobacter*); (ii) aerobic gram-positive bacteria (*Cellulomonas* and *Thermobifida*); and (iii) aerobic gliding bacteria (*Cytophaga*, and *Sporocytophaga*). Generally, only a few species within each of the above-named genera are actively cellulolytic. The distribution of cellulolytic capability among organisms differing in oxygen relationship, temperature, and salt tolerance is a testament to the wide availability of cellulose across natural habitats. Complicating the taxonomic picture is the recent genomic evidence that the noncellulolytic solventogenic *Clostridium acetobutylicum* contains a complete cellulosomal gene cluster system that is not expressed, due in part to disabled promoter sequences (606). Examination of the rapidly expanding genomics database may reveal similar surprises in the future.

Among the bacteria, there is a distinct difference in cellulolytic strategy between the aerobic and anaerobic groups. With relatively few exceptions (549, 659), anaerobes degrade cellulose primarily via complexed cellulase systems exemplified by the well-characterized polycellulosome organelles of the thermophilic bacterium *Clostridium thermocellum* (606). Cellulolytic enzymes in *C. thermocellum* cultures are typically distributed both in the liquid phase and on the surface of the cells. However, several anaerobic species that utilize cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complexed cellulases directly on the surface of the cell or the cell-glycocalyx matrix. Most anaerobic cellulolytic species grow optimally on cellulose when attached to the substrate, and in at least a few species this adhesion appears to be obligate. Cellulolytic anaerobes resemble other fermentative anaerobes in that their cell yields are low, with the bulk of substrate being converted to various fermentation end products, including ethanol, organic acids, CO_2, and H_2.

Aerobic cellulose degraders, both bacterial and fungal, uti-

TABLE 1. Major morphological features of cellulolytic bacteria

Oxygen relationship	Genus	Representative species[a]	Gram reaction	Morphology	Growth temp.[b]	Resting state	Motility	Features of cellulase system	References
Aerobic	*Acidothermus*	*A. cellulolyticus*	+	Rod	Thermo			Noncomplexed, cell free	48
	Bacillus	*B. pumilis*	+	Rod	Meso	Endospore	Flagellar	Noncomplexed, cell free	220
	Caldibacillus	*C. cellovorans*	+	Rod	Thermo	Endospore		Noncomplexed, cell free	48
	Cellulomonas[c]	*C. flavigena, C. uda*	+	Rod	Thermo	None	Flagellar	Noncomplexed, cell free	25, 26, 493
	Cellvibrio	*C. fulvus, C. gilvus*	−	Curved rod	Meso	None	Flagellar	Noncomplexed, cell free	612
	Cytophaga	*C. hutchinsonii*	−	Rod	Meso	None	Gliding	Noncomplexed?, cell free?	322, 384
	Erwinia	*C. carotovora*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free	29
	Micromonospora	*M. chalcae*	+	Filamentous rod	Meso	Spore[d]	Nonmotile	Noncomplexed, cell free	200, 215
	Pseudomonas	*P. fluorescens* var. *cellulosa*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free	331
	Sporocytophaga	*S. myxococcoides*	−	Rod	Meso	Spore[d]	Gliding	Noncomplexed, cell free	697
	Rhodothermus	*R. marinus*		Rod	Thermo				11, 48
	Streptomyces	*S. reticuli*	+	Filamentous rod	Meso	Spore[d]	Nonmotile	Noncomplexed, cell free	715
	Thermobifida	*T. fusca*	+	Filamentous rod	Thermo	Spore[d]	Nonmotile	Noncomplexed, cell free	777
Anaerobic	*Acetivibrio*	*D. cellulolyticus*	−	Curved rod	Meso	None	Nonmotile	Complexed	327, 387, 589
	Anaerocellum	*D. thermophilum*	+	Rod	Thermo	None	Flagellar	Noncomplexed, cell free	659
	Butyrivibrio	*B. fibrisolvens*	+	Curved rod	Meso	None	Flagellar	Noncomplexed	294
	Caldicellulosiruptor	*C. saccharolyticum*	−	Rod	Thermo	None	Flagellar	Noncomplexed, cell free	549
	Clostridium	*C. thermocellum, C. cellulolyticum*	+	Rod	Thermo, meso	Endospore	Flagellar	Complexed, mostly cell bound[e]	392, 415, 485, 532, 613
	Eubacterium	*E. cellulosolvens*	+	Rod	Meso	None	Nonmotile		699
	Fervidobacterium	*F. islandicum*	+	Rod	Thermo	None	Flagellar		292
	Fibrobacter	*F. succinogenes*	−	Rod	Meso	None	Nonmotile	Complexed, cell bound	88, 294, 463, 645
	Halocella	*H. cellulolytica*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free	622
	Ruminococcus	*R. albus, R. flavefaciens*	+	Coccus	Meso	None	Nonmotile	Complexed, cell bound	88, 294
	Spirochaeta	*S. thermophila*	+	Spiral	Thermo	None		Noncomplexed, cell free	8, 48
	Thermotoga	*T. neapolitana*	−	Rod	Thermo				48

[a] Not all strains of the indicated species are cellulolytic, and some less active or less studied cellulolytic species within these genera are not listed.
[b] Meso, mesophilic; Thermo, themophilic.
[c] Most strains can also grow anaerobically.
[d] Unlike true endospores, these spores have only moderate resistance to environmental stress.
[e] Except for *C. stercorarium* (81, 83, 606).

lize cellulose through the production of substantial amounts of extracellular cellulase enzymes that are freely recoverable from culture supernatants (554, 606), although enzymes are occasionally present in complexes at the cell surface (67, 715). The individual enzymes often display strong synergy in the hydrolysis of cellulose. While many aerobic bacteria adhere to cellulose, physical contact between cells and cellulose does not appear to be necessary for cellulose hydrolysis. Kauri and Kushner (322) have shown that separating *Cytophaga* cells from cellulose via an agar layer or membrane filters appears to enhance cellulose utilization; they suggest that this separation may dilute hydrolytic products, thus relieving catabolite repression of enzyme synthesis. Aerobic cellulolytic bacteria and fungi produce high cell yields characteristic of aerobic respiratory growth, and this has led to considerable technological interest in producing microbial cell protein from waste cellulosic biomass (175, 567, 594, 623). In addition, many studies of aerobic cellulolytic microbes have focused on improving the yield and characteristics of cellulase enzymes. The physiology of the organisms themselves has received surprisingly little study, apart from studies on the effect of growth conditions on enzyme secretion (see, e.g., reference 236).

An interesting point suggested from Table 1 is that cellulose utilization generally proceeds via organisms that are either aerobic or anaerobic, but not both. Indeed, despite the wide distribution of facultatively anaerobic bacteria in general, members of the genus *Cellulomonas* are the sole reported facultatively anaerobic cellulose degraders (25, 26, 113, 150). Whether the general paucity of facultatively anaerobic groups is a consequence of a physiological or ecological incompatibility of two fundamentally different strategies for cellulose utilization employed by the two groups remains an interesting open question.

It is also notable that most aerobic cellulolytic bacterial species common in soil are classified within genera well known for secondary (non-growth-associated) metabolism, including the formation of distinct resting states (*Bacillus*, *Micromonospora*, and *Thermobifida*) and/or production of antibiotics (*Bacillus* and *Micromonospora*) and other secondary metabolites. While antibiotic production in cellulolytic species has not been systematically investigated, production of such compounds might provide additional selective fitness to compensate for their rather modest maximum growth rate on cellulose. An ability to form resting states relatively resistant to starvation or other environmental insult also provides a selective advantage in nature.

Cellulase Enzyme Systems

As noted in the discussion of structure and composition (see above), natural cellulosic substrates (primarily plant cell materials) are composed of heterogeneous intertwined polysaccharide chains with varying degrees of crystallinity, hemicelluloses and pectins, embedded in lignin. Microorganisms produce multiple enzymes to degrade plant cell materials, known as enzyme systems (722). Although this discussion focuses primarily on the action of hydrolytic enzyme systems on cellulose, it should be realized that such systems are also active on hemicellulose, and enzymes active specifically on hemicellulose are commonly coproduced by cellulolytic microorganisms. Prior reviews consider the complexed cellulases of anaerobic bacteria (31, 33, 36, 37, 38, 39, 40, 41, 43, 165, 166, 182, 383, 565, 606, 621), noncomplexed fungal and bacterial cellulases (122, 349, 579, 653, 672, 673, 756), cellulase structure and catalytic mechanisms (58, 136, 470, 624, 683, 722, 757, 758), cellulase (hydrolase) families (254, 255, 256, 258, 260), and biotechnological applications (52, 214, 501, 604, 605).

For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated. The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past two decades. Components of cellulase systems were first classified based on their mode of catalytic action and have more recently been classified based on structural properties (260). Three major types of enzymatic activities are found: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (672). β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Fig. 1). Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end (58, 751).

The insoluble, recalcitrant nature of cellulose represents a challenge for cellulase systems. A general feature of most cellulases is a modular structure often including both catalytic and carbohydrate-binding modules (CBMs). The CBM effects binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate, insoluble cellulose. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases (673) (Fig. 1A). Revisiting the original model of cellulose degradation proposed by Reese et al. (563), a possible additional noncatalytic role for CBMs in cellulose hydrolysis was proposed: the "sloughing off" of cellulose fragments from cellulosic surfaces of, e.g., cotton fibers, thereby enhancing cellulose hydrolysis (161). Cellulase systems exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between

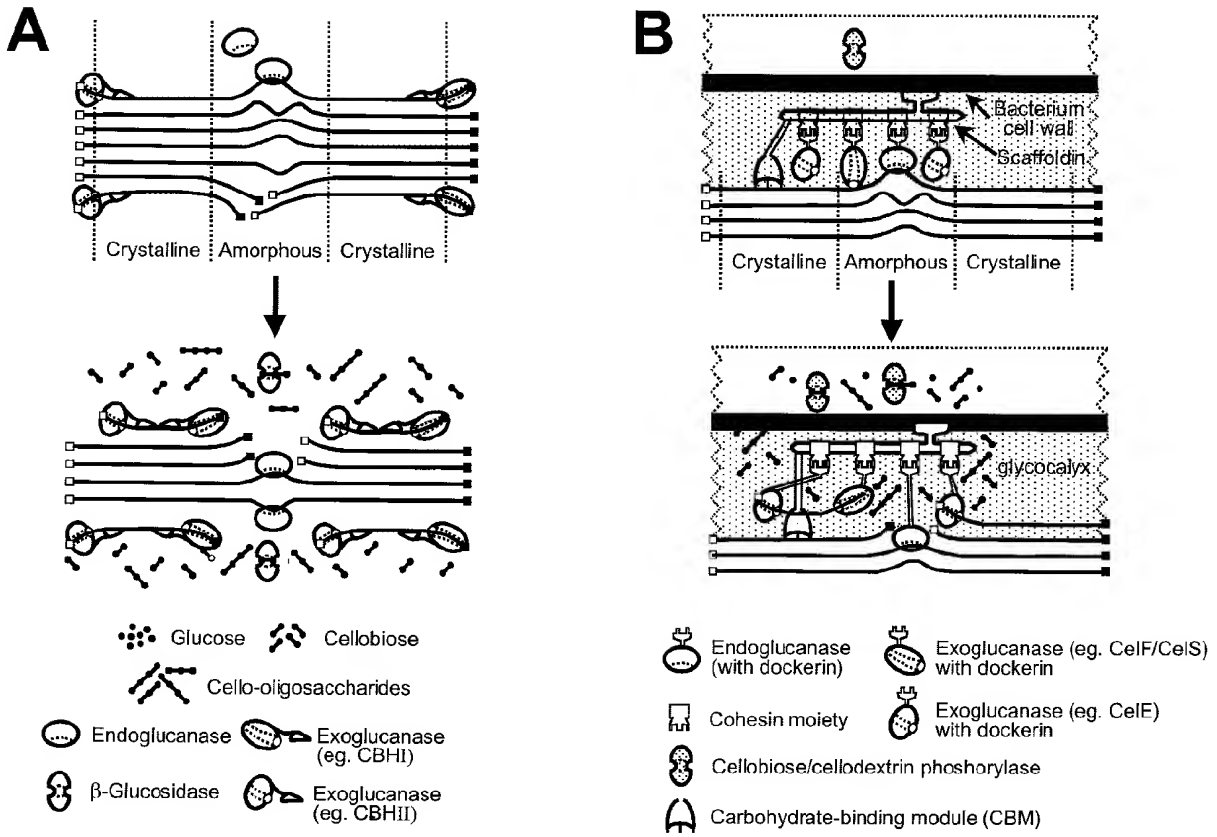

FIG. 1. Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by noncomplexed (A) and complexed (B) cellulase systems. The solid squares represent reducing ends, and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale.

exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs (161, 672).

Cellulase systems are not merely an agglomeration of enzymes representing the three enzyme groups (endoglucanases, exoglucanases, and β-glucosidases, with or without CBMs), but rather act in a coordinated manner to efficiently hydrolyze cellulose. Microorganisms have adapted different approaches to effectively hydrolyze cellulose, naturally occurring in insoluble particles or imbedded within hemicellulose and lignin polymers (683). Cellulolytic filamentous fungi (and actinomycete bacteria) have the ability to penetrate cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles (176). The production of "free" cellulases, with or without CBMs, may therefore suffice for the efficient hydrolysis of cellulose under these conditions. The enzymes in these cellulase systems do not form stable high-molecular weight complexes and therefore are called "noncomplexed" systems (Fig. 1A). By contrast, anaerobic bacteria lack the ability to effectively penetrate cellulosic material and perhaps had to find alternative mechanisms for degrading cellulose and gaining access to products of cellulose hydrolysis in the presence of competition from other microorganisms and with limited ATP available for cellulase synthesis. This could have led to the development of "complexed" cellulase systems (called "cellulosomes"), which position cellulase-producing cells at the site of hydrolysis, as observed for clostridia and ruminal bacteria (Fig. 1B). Noncomplexed cellulase systems are discussed first, highlighting the cellulase systems of the aerobic filamentous fungi *Trichoderma reesei* and *Humicola insolens* as well as aerobic actinomycetes belonging to the genera *Cellulomonas* and *Thermobifida*. The interesting multidomain cellulase systems of anaerobic hyperthermophilic bacteria are mentioned briefly. Thereafter the complexed cellulase systems of anaerobic *Clostridium* species, *Ruminococcus* species, and anaerobic fungi are considered.

Noncomplexed cellulase systems. Cellulases from aerobic fungi have received more study than have those of any other physiological group, and fungal cellulases currently dominate the industrial applications of cellulases (235, 492, 614). In particular, the cellulase system of *T. reesei* (teleomorph:*Hypocrea jecorina*, initially called *Trichoderma viride*) has been the focus of research for 50 years (424, 561, 562, 563). *T. reesei* produces at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two β-glucosidases (BGLI and BGLII (358, 494, 664). Intensive efforts over several decades to enhance cellulase yields have resulted in strains that produce up to 0.33 g of protein/g

US006350601B1

(12) **United States Patent**
Aoki et al.

(10) **Patent No.: US 6,350,601 B1**
(45) **Date of Patent: *Feb. 26, 2002**

(54) **ENZYME COMPOSITION FOR USE AS A CLINICAL DIAGNOSTIC REAGENT**

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(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/836,833**

(22) PCT Filed: **Nov. 10, 1995**

(86) PCT No.: **PCT/JP95/02303**

§ 371 Date: **May 8, 1997**

§ 102(e) Date: **May 8, 1997**

(87) PCT Pub. No.: **WO96/15256**

PCT Pub. Date: **May 23, 1996**

(30) **Foreign Application Priority Data**

Nov. 11, 1994 (JP) 6-277617

(51) **Int. Cl.**[7] **C12N 9/96**; C12N 9/00

(52) **U.S. Cl.** **435/188**; 435/15; 435/17; 435/18; 435/21; 435/25; 435/26; 435/183; 435/196; 435/194; 435/193; 435/190; 435/212; 436/8; 436/15; 436/16; 436/18; 530/350; 530/362; 530/363; 424/94.5; 424/94.6; 424/94.64; 424/94.63

(58) **Field of Search** 435/15, 17, 18, 435/212, 21, 190, 25, 193, 26, 188, 196; 436/8, 15, 16, 18; 530/350, 362, 363; 429/94.5, 94.6, 94.64, 94.63

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(57) **ABSTRACT**

Disclosed is a stabilized enzyme composition for use in clinical examination, comprising: (a) an enzyme component comprising at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase; (b) a stabilizer component comprising effective stabilizing amounts of an albumin, and at least one saccharide selected from the group consisting of trehalose and sorbitol; and (c) an aqueous medium having dissolved therein the components (a) and (b). The enzyme composition of the present invention is stable for a prolonged period of time not only under non-freeze refrigeration conditions, but also under freezing conditions or under conditions for non-freeze refrigeration after thawing of the frozen composition, as compared to the conventional enzymatic compositions. The enzyme composition of the present invention can be advantageously used for the purpose of checking the precision in measurement, correcting measured values and calibrating the amount and activity of an enzyme, in a clinical examination for measuring the enzymatic activity in a sample, such as serum or the like.

6 Claims, No Drawings

ENZYME COMPOSITION FOR USE AS A CLINICAL DIAGNOSTIC REAGENT

FIELD OF THE INVENTION

The present invention relates to a novel enzyme composition for use in clinical examination. More particularly, the present invention is concerned with a stabilized enzyme composition for use in clinical examination, comprising (a) an enzyme component comprising at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase; (b) a stabilizer component comprising effective stabilizing amounts of an albumin, and at least one saccharide selected from the group consisting of trehalose and sorbitol; and (c) an aqueous medium having dissolved therein the components (a) and (b).

The enzyme composition of the present invention is stable for a prolonged period of time not only under non-freeze refrigeration conditions, but also under freezing conditions or under conditions for non-freeze refrigeration after thawing of the frozen composition, as compared to conventional enzyme compositions. Specifically, with respect to each of at least two enzymes contained in the enzyme composition of the present invention, the activity can be maintained, for a prolonged period of time, in the liquid state or in the frozen state (i.e., in a non-lyophilized state). Therefore, the enzyme composition of the present invention is advantageous from the economical viewpoint and from the viewpoint of ease in handling.

In a clinical examination for measuring the enzymatic activity in a sample, such as serum or the like, the enzyme composition of the present invention can be advantageously used for the purpose of checking the precision in measurement, correcting measured values and calibrating the activity of an enzyme in the sample.

PRIOR ART

In clinical examinations, for measuring the enzymatic activity in a sample, such as serum or the like, enzyme compositions are used for the purpose of checking the precision in measurement, correcting. measured values and calibrating the amount and activity of an enzyme in the sample. At present, as such enzyme compositions, various compositions containing a single type of enzyme or containing an enzyme system comprising a plurality of types of enzymes which participate in consecutive reactions are commercially available. These enzyme compositions are used as control serum for checking the precision in measurement and as reference materials (standard materials) for rectifying differences between measured values obtained in different testing facilities, and also as calibrators for calibrating the amount and activity of enzymes. [With respect to the terms "control serum", "reference material" and "calibrator", which will be further explained below, reference can be made, for example, to "Kensa-to-Gijutsu (MODERN MEDICAL LABORATORY)", vol. 22, No. 8, p. 594, 1994]. Such an enzyme composition is prepared by adding the same enzyme as the enzyme to be determined to a serum product, such as pooled human serum, pooled animal serum, human albumin, bovine serum albumin or the like.

As the enzyme to be added to a serum product in order to prepare an enzyme composition, various enzymes derived from human sources and animal sources have been reported. For example, as control sera, "Monitrol" and "Monitrol L" (registered trade marks; both manufactured and sold by

International Reagents Corporation, Japan), each of which is produced by adding an enzyme from animal sources to pooled human serum, are commercially available as a lyophilized product and as a frozen product, respectively. As examples of commercially available reference materials, there can be mentioned "SERACLEAR-HE" (trade name; manufactured and sold by NIPPON SHOJI KAISHA, Ltd., Japan) which is produced by adding an enzyme from established human cell lines or human source (erythrocyte) to pooled human serum, and "Enzyme reference" which is produced by adding the above-mentioned human-derived enzyme to bovine serum albumin. Among these commercially available enzyme compositions, with respect to those containing pooled human serum as a serum product, there is a danger that they have been contaminated with a known virus (such as HIV) or an unknown virus. Therefore, in use of such enzyme compositions containing pooled human serum, minute care is required for preventing biohazard.

The enzymatic activity of each of these enzyme compositions is determined by using an enzymatic assay reagent specific for the enzyme in the enzyme composition. Generally, in the measurement of a catalytic activity, such as an enzymatic activity, the measured value greatly varies depending on not only the type and concentration of the substrate, but also the reaction conditions, such as pH and reaction temperature.

In judging the condition of a patient by utilizing the enzymatic activity in a sample from the patient, such as serum or the like, wherein the enzymatic activity is measured by a diagnostic reagent, the above-mentioned fact that the measured value of enzymatic activity varies depending on the measuring conditions poses a serious problem. Further, various kits of reagents for measurement of an enzymatic activity are sold by many manufacturers. When a plurality of samples from the same lot are individually measured by using a plurality of reagent kits wherein the measuring conditions are greatly different among the measurements, largely different measured values are obtained with respect to the samples from the same lot, thus causing a confusion in the diagnosis.

In order to solve the above-mentioned problem, socalled "recommended methods", each of which prescribes detailed measuring conditions agreed to by many scientists, have been proposed by scientific societies. For example, there can be mentioned a recommended method proposed by the International Federation of Clinical Chemistry (IFCC) and a recommended method proposed by the Japan Society of Clinical Chemistry (JSCC). However, such recommended methods have a problem in that they do not use an automatic analyzer and, therefore, cannot be used in a testing facility which has to deal with a large number of samples in a day. Accordingly, for enabling the accuracy of such a recommended method to be directly reflected in the test results obtained by using an automatic analyzer, it becomes necessary to use a reference material which has been measured with respect to the enzymatic activity thereof by using the recommended method. Specifically, a measured value obtained routinely by means of an automatic analyzer using commercially available reagents can be corrected based on a standard value obtained by the measurement of a reference material using a recommended method, so that the enzymatic activity of a sample can be accurately measured to obtain a reliable value which does not depend on the measuring conditions. Thus, by using a reference material, differences among measured values obtained by different testing facilities can be suppressed to a minimum.

As explained above, a reference material is used for reflecting the accuracy of a recommended method in a

routine measuring method. Therefore, it is required that a reference material have the same properties as those of a human-derived sample. Recently, from the viewpoint of achieving the interchangeability of test data, the importance of reference materials have been greatly recognized, and a reference material produced using a human-derived enzyme has also been commercially available. There exist a wide variety of human-derived enzymes. Some human-derived enzymes assume the form of isozymes, for example, derived from different organs, which isozymes are identical to one another in the enzymatic function but are different from one another in characteristics, such as specificity to organs. Further, the content of an enzyme in human-derived serum varies depending on the individual as a source of the serum and on the disease condition of the individual as a source of serum. For example, it is well known that there are various types of alkaline phosphatases which are derived from placenta, small intestine and a tumor cell, and these various types of alkaline phosphatases exhibit largely different levels of activity depending on the type of the buffer solution which is used for adding the enzyme thereto.

On the other hand, control serum is generally used for checking the precision of the measurement of the enzymatic activity in a sample. Specifically, control serum is used for checking (based on the measured values obtained by the measurement using the control serum) whether or not reagents and equipment perform normal functions. Accordingly, with respect to control serum, rather than the level of the enzymatic activity, it is important that the enzymatic activity of the control serum does not change for the period of time during which the measurement by using the control serum is conducted.

On the other hand, a calibrator is used for determining a calibration factor for an enzyme to be tested. The calibrator is also used for checking the precision of the measurement of the enzymatic activity by a specific reagent for enzymatic activity. This means that the calibrator may function as control serum. Further, a material which is used for the calibration of the enzymatic activity of an enzyme may also be a reference material for the enzyme. Thus, in respect of the function and role in the measurement of enzymatic activity, it is considered that a calibrator is positioned between control serum and a reference material.

These enzyme compositions are usually sold in the form of a lyophilized product, a liquid product or a frozen product.

A lyophilized product has excellent storage stability, but has problems in that a denaturation of lipoprotein occurs during the lyophilization, so that a solution obtained by dissolving the lyophilized product in water or the like is likely to become turbid, and an error in measuring the volume is likely to occur. Further, it has frequently been observed that the activity of an enzyme contained in a lyophilized product changes at the dissolution of the lyophilized product. For example, it has been reported that, with respect to a lyophilized product, large differences in activity are observed between vials of a single lot [see "Kensa-to-Gijutsu (MODERN MEDICAL LABORATORY)", vol. 20, No. 12, p. 1041, 1992]. More illustratively, especially in the case of alkaline phosphatase, a serious problem is likely to occur. For example, it has been pointed out that alkaline phosphatase is reversibly deactivated by serum lipoprotein. Further, it has been observed that, when a commercially available lyophilized control serum is stored at 25° C. after being dissolved in water or the like, the alkaline phosphatase present in the serum exhibits a considerable increase in activity within 24 hours from the dissolution [see "Seibutsu-Siryo-Bunseki (Journal of Analytical Bio-Science)", vol. 14, No.2, 1991]. Thus, with respect to alkaline phosphatase, there has been no lyophilized product which is satisfactory in respect of the suppression of a change in the alkaline phosphatase activity after dissolution. Further, it has also been reported that the temperature of a liquid (usually, water) used for dissolution affects the activity of an enzyme. For example, with respect to a lyophilized enzyme composition containing creatine kinase, it has been reported that a solution obtained by dissolving it in a dissolution liquid at 2 to 8° C. exhibits a creatine kinase activity higher than that of a solution obtained by dissolving it in a dissolution liquid at room temperature [see "Rinsyo-Kensa-Kiki-Shiyaku (The Journal of Clinical Laboratory, Instruments and Reagents)" vol. 15, No. 4, 1992]. From these reports, it can be concluded that the period during which a solution obtained by dissolving a lyophilized enzyme composition in a dissolution liquid is stable is as short as one or two days.

By contrast, with respect to a product in liquid or frozen state, it is not necessary to dissolve the product in a dissolution liquid (usually, water), so that the operation before usage is relatively easy, and an error in a dissolution operation does not occur. Further, the problem of the change in enzymatic activity at dissolution, which is disadvantageously large in the case of a lyophilized product, is eliminated. In using a frozen product, a thawing operation is necessary, whereas a product in liquid state can be used as it is, so that the use of products in liquid state has recently been increased also in the field of clinical biochemical examinations. A product in liquid state is also called a "reagent usable without reconstitution", since it can be used without a preparatory operation, such as thawing, dissolution or the like. Reagents usable without reconstitution enable simplification of operations and saving of man power in biochemical examination facilities.

Therefore, in the future, enzyme compositions for use in clinical examinations, such as control serum, a reference material, a calibrator and the like, would necessarily be provided as reagents usable without reconstitution. However, with respect to the above-mentioned enzyme compositions for use in clinical examinations, at the current technological level, it is impossible to provide them in the form of reagents usable without reconstitution. The only form of an enzyme composition which is practically usable at present is the frozen form. Moreover, with respect to a frozen product containing alkaline phosphatase, it is reported that a gradual increase in enzymatic activity is observed after thawing [see "Kensa-to-Gijutsu (MODERN MEDICAL LABORATORY)", vol. 20, No. 12, p. 1039, 1992]. Also, with respect to an enzyme composition containing creatine kinase frozen at −20° C., it has been reported that a decrease in the activity of creatine kinase is observed when the freeze storage period exceeds 1 to 3 months [see "Kensa-to-Gijutsu (MODERN MEDICAL LABORATORY)", vol. 21, No. 5, extra issue, 1993]. Thus, frozen products available at present are not always satisfactory.

In clinical examinations, alkaline phosphatase is useful not only as a tumor marker, but also as means for obtaining much information on the condition of disease. Creatine kinase has 3 isozymes and is extremely localized in specific organs, as compared to enzymes, such as alanine aminotransferase, aspartate aminotransferase and the like, which are widely used in clinical examinations at present. Most creatine kinase is present in skeletal muscles, cardiac muscle, smooth muscle and the brain. Accordingly, by determining creatine kinase, identification of injury of cre-

atine kinase-related organs would be able to be made. Therefore, it is desired to develop a stabilized enzyme composition containing at least alkaline phosphatase and creatine kinase, especially in a liquid form or a frozen form, which can be used as control serum, a reference material or a calibrator.

In general, an enzyme is a protein and, therefore, a solution of an enzyme is unstable. As a method for obtaining an enzyme having improved stability, a method is known in which a thermophilic microorganism is cultured and then, an enzyme is obtained from the resultant culture of the thermophilic microorganism. This method is effective because thermophilic microorganisms produce thermostable enzymes. In recent years, it has become possible to design or modify a thermostable enzyme, using the techniques of protein engineering, based on the information on the genes, amino acids, steric structures and the like of proteins. On the other hand, for improving the stability of an enzyme, various methods have been proposed in which the composition of a solution containing the enzyme is adjusted or modified.

For example, a polyol, such as glycerol, is known as a generally employed stabilizing agent. However, a vast plurality of types of enzymes are known, and their properties, such as optimum pH and the like, are also diversified. A stabilizing agent which is effective for all of the enzymes has not been reported. On the other hand, various reports have been made on the method for stabilizing a single specific enzyme.

For example, a creatine kinase composition having improved stability, which is obtained by using as a stabilizing agent a protein having an unreactive sulfhydryl group, is disclosed in Unexamined Japanese Patent Application Laid-Open Specification No. 5-207880 (corresponding to U.S. Pat. No. 5,217,890). Further, U.S. Pat. No. 5,298,406 discloses a creatine kinase composition having improved stability which comprises ascorbic acid and non-reducing polyol as stabilizers (antioxidants) and an aminoglycoside antibiotic as an antimicrobial agent. All of the above-mentioned stabilizers are intended to stabilize the SH group which is essential for the activity of creatine kinase.

It has long been believed that, when a composition contains two or more enzymes, such as creatine kinase and alkaline phosphatase, it is difficult to find out conditions effective for stabilizing all of the enzymes in the composition, since the conditions effective for stabilizing an enzyme frequently render another enzyme unstable. For example, a magnesium ion is not only necessary for activating alkaline phosphatase [see "Koso Handobukku (Enzyme Handbook)", p 434, published in 1983 by Asakura Shoten, Japan], but also functions as a stabilizer for alkaline phosphatase. However, on the other hand, it is well known that the storage stability of creatine kinase is increased by the addition of a chelating reagent, which coordinates to a metal ion, such as magnesium ion, to form a chelate with the metal ion (see Clin. Chem., 23, 1119, 1977). Thus, a stabilizing agent for alkaline phosphatase and that for creatine kinase are opposite or incompatible to each other in properties. Further, since creatine kinase is an SH enzyme as described above, it is stabilized in the presence of a thiol compound, such as cysteine, mercaptoethanol, N-acetylcysteine, cysteinyl-bovine serum albumin described in the above-mentioned Unexamined Japanese Patent Application Laid-Open Specification. On the other hand, the presence of cysteinyl-bovine serum albumin renders alkaline phosphatase unstable, and, as shown in Example 1 below, N-acetylcysteine also renders alkaline phosphatase unstable.

Still further, it has been reported that the conditions effective for the stabilization of an enzyme composition in a liquid form is generally different from those effective for the stabilization of the enzyme composition in a frozen form, and therefore it is difficult to find out conditions under which an enzyme composition is stabilized either in a liquid form or in a frozen form. For example, the freeze storage stability of alkaline phosphatase is lowered by the addition of sucrose (see Example 5 of the present application). It has also been reported that the stability of lactate dehydrogenase is rather decreased at a low temperature [see Rinsho Kagaku (Clinical Chemistry) vol. 19, No. 2, 1990], despite the fact that an enzyme is generally known to be more stable at a lower temperature.

In some cases of clinical examination, the activity of an enzyme (first enzyme) is measured utilizing another enzyme (second enzyme) which participates in the enzyme reaction together with the first enzyme. In recent years, a reagent kit for measurement of enzymatic activity, in which the above-mentioned second enzyme is contained in a stabilized form, has been developed as a kit of reagents usable without reconstitution. In the case of such an enzyme composition, the stability of the enzymatic activity is not very important as long as the enzymatic activity is maintained to some extent. Therefore, the enzyme may be contained in an excess amount in order to make up for a lowering of the enzymatic activity. However, with respect to an enzyme composition for use in a clinical examination for the purpose of checking the precision in measurement, correcting measured values and calibrating the amount or enzymatic activity of an enzyme, such as a reference material, a calibrator or control serum, the stability of the enzymatic activity is a very important factor influencing the commercial value of the enzyme composition. The stabilization of an enzyme composition for use in checking the precision in measurement, correcting measured values and calibrating the amount or enzymatic activity of an enzyme is very difficult not only for the reasons described above but also because the concentration of the enzyme is very small.

In these situations, it has been desired to develop a stabilized enzyme composition containing at least two enzymes (at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase), wherein each of the enzymes exhibits almost no lowering of activity during storage, which enzyme composition can be advantageously used for checking the precision in measurement, correcting measured values and calibrating the amount or enzymatic activity of an enzyme.

SUMMARY OF THE INVENTION

In these situations, the present inventors have made extensive and intensive studies with a view toward developing a stabilized enzyme composition which contains at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase which are important in clinical examination, which composition can be advantageously used in clinical examination for checking of the precision in measurement, correction of a measured value, and/or calibration. As a result, it has unexpectedly been found that, when a combination of a specific saccharide and an albumin is used as a stabilizer for an enzyme composition, all of the enzymes contained in the enzyme composition can maintain their respective activities for a prolonged period, not only in a liquid form without experiencing freezing, but also in a frozen form and in a liquid form after thawing of the once

frozen product, as compared to a conventional enzyme composition containing only one enzyme which is commercially available and is used for the same purpose as mentioned above. The present invention has been completed, based on the above finding.

Therefore, it is a primary object of the present invention to provide an enzyme composition for use in clinical examination, containing at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase, wherein the activities of all of the enzymes contained in the enzyme composition are stabilized, so that the composition is not only advantageous from an economical viewpoint, but also useful as control serum, a reference material or a calibrator which enables extremely reliable measurement with very small variation in measured values, as compared to the conventional enzyme compositions containing only one enzyme.

DETAILED DESCRIPTION OF THE INVENTION

Essentially, in the present invention, there is provided a stabilized enzyme composition for use in clinical examination, comprising:

(a) an enzyme component comprising at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase;

(b) a stabilizer component comprising effective stabilizing amounts of an albumin, and at least one saccharide selected from the group consisting of trehalose and sorbitol; and

(c) an aqueous medium having dissolved therein the components (a) and (b).

By virtue of the presence of a stabilizer component comprising an albumin, and at least one saccharide selected from the group consisting of trehalose and sorbitol, the enzyme composition of the present invention exhibits excellent stability, with respect to the activities of all of the enzymes contained in the composition, not only under freezing conditions but also under non-freeze refrigeration conditions, irrespective of whether or not the composition has an experience of being frozen.

In an essential aspect of the present invention, the enzyme component of the enzyme composition comprises a plurality of enzymes, namely, at least two enzymes selected from the group consisting of alkaline phosphatase (ALP) (EC.3.1.3.1), creatine kinase (CK) (EC.2.7.3.2) and alanine aminotransferase (ALT) (EC.2.6.1.2), differing from the conventional enzyme composition containing a single enzyme. Representative examples of combinations of at least two enzymes include a combination of two enzymes ALP and CK, and a combination three enzymes ALP, CK and ALT.

Further, in the present invention, it is preferred that the enzyme component of the enzyme composition further comprises at least one additional enzyme selected from the group consisting of aspartate aminotransferase (AST) (EC.2.6.1.1), lactate dehydrogenase (LDH) (EC.1.1.1.27) and γ-glutamyl transpeptidase (γ-GTP) (EC.2.3.2.2). Representative examples of at least one additional enzyme include AST singly used, and a combination of three enzymes AST, LDH and γ-GTP.

It is most preferred that the enzyme component of the enzyme composition of the present invention comprises ALP, CK and ALT, as enzyme component (a), and AST, LDH and γ-GTP as additional enzymes.

When the composition of the present invention comprises the components described in the above-mentioned essential aspect of the present invention, it is preferred that, when selected as a constituent of the enzyme component (a), the alkaline phosphatase is present in a concentration of from 9 to 6500 U, the creatine kinase is present in a concentration of from 6 to 4000 U, and the alanine aminotransferase is present in a concentration of from 3 to 1150 U, each per liter of the composition.

With respect to the effective stabilizing amount of the stabilizer component (b), it is preferred that the albumin is present in a concentration of from 0.3 to 7 (w/v) %, and the at least one saccharide selected from the group consisting of trehalose and sorbitol is present in a concentration of from 2 to 15 (w/v) %, each based on the volume of the composition.

Examples of albumins used as a part of the stabilizer component include an albumin obtained from a mammal, such as human serum albumin and bovine serum albumin (BSA), and an albumin obtained from a bird, such as chick serum albumin. As these albumins, commercially available albumins can be used. It is preferred that the concentration of the albumin in the enzyme composition of the present invention is from 0.3 to 7 (w/v) %, more preferably from 1 to 5 (w/v) %.

At least one saccharide used as a part of the stabilizer component is selected from the group consisting of trehalose and sorbitol. It is preferred that the concentration of the at least one saccharide in the enzyme composition of the present invention is from 2 to 15 (w/v) %, more preferably from 3 to 10 (w/v) %. For controlling the viscosity of the composition with ease and for achieving a satisfactory stabilizing effect, the concentration of the at least one saccharide is preferred to fall within the range mentioned above. If desired, the at least one saccharide can be a mixture of sorbitol and trehalose.

When the enzyme component (a) further comprises the at least one additional enzyme selected from the group consisting of aspartate aminotransferase, lactate dehydrogenase and γ-glutamyl transpeptidase, it is preferred that, when selected as the additional enzyme, the aspartate aminotransferase is present in a concentration of from 3 to 1150 U, the lactate dehydrogenase is present in a concentration of from 8 to 4000 U, and the γ-glutamyl transpeptidase is present in a concentration of from 2 to 1200 U, each per liter of the composition. If desired, amylase, lipase or the like can be added to the composition. It is preferred that, when added to the composition, amylase is present in a concentration of from 25 to 1000 U, and lipase is present in a concentration of from 5 to 1000 U, each per liter of the composition.

With respect to the activity of an enzyme, the amount of the enzyme required to convert 1 μmol of the substrate at 37° C. is defined as 1 U. For example, with respect to each of ALP, CK, ALT, AST and LDH, the activity thereof can be determined by the consensus method which is the same method as the recommended method proposed by the Japanese Society of Clinical Chemistry ["Rinsho Kagaku (Japanese Journal of Clinical Chemistry)", vol. 19, p.184 and p.209 (1990); ivid, vol. 18, p.211 and p.226 (1989); ivid, vol. 19, p.228 (1990)], except that the measurement temperature is changed from 30° C. to 37° C., or can be determined by using commercially available reagents (such as reagents used in the Examples mentioned below) for the determination of the enzymatic activity of a respective enzyme. The activity of γ-GTP can be determined by using a commercially available kit for the determination of the activity of the enzyme ('Determiner γ-GTP', manufactured

and sold by KYOWA MEDEX Co., Ltd., Japan), which kit uses γ-glutamyl-3,5-dibromo-4-hydroxyanilide as a substrate. This substrate is converted by the action of γ-GTP to 3,5-dibromo-4-hydroxyanilide (DBHA), and the generated DBHA is oxidatively condensed with 1-N-ethyl-N-(3-methylphenol)-N-succinyl ethylenediamine (EMSE) by the action of a monophenol monooxygenase (MPO), such as ascorbate oxydase, laccase or the like, to thereby generate a green condensate exhibiting an absorption at a wavelength of 710 nm. The activity of γ-GTP can be determined by calorimetric determination of the condensate.

With respect to the origin of the enzymes used for producing the enzyme composition of the present invention, there is no particular limitation as long as the enzymes are suitable as a component for an enzyme composition which is provided in a liquid form or frozen form. However, it is preferred that each of the enzymes is derived from an animal, more preferably from human.

With respect to the enzymes derived from an animal, examples of alkaline phosphatase (ALP) include bovine kidney ALP (catalog No. P6680, Sigma Chemical Company, U.S.A.), bovine intestinal ALP (catalog No. P0280, Sigma Chemical Company, U.S.A.), hog kidney ALP (catalog No. P4439, Sigma Chemical Company, U.S.A.), chick intestinal ALP (catalog No. P8008, Sigma Chemical Company, U.S.A.) and the like. Examples of creatine kinase (CK) include bovine heart CK (catalog No. C7886, Sigma Chemical Company, U.S.A.), hog heart CK [tradename: 'Monitrol (L)', International Reagents Corporation, Japan], chick heart CK (tradename: 'Control WAKO', Wako Pure Chemical Industries Ltd., Japan), rabbit muscle CK (catalog No. C3755, Sigma Chemical Company, U.S.A.) and the like. Examples of aspartate aminotransferase (AST) include bovine heart AST (tradename: 'Monitrol II', International Reagents Corporation, Japan), hog heart AST (catalog No. G2751, Sigma Chemical Company, U.S.A.) and the like. Examples of alanine aminotransferase (ALT) include bovine heart ALT (tradename: 'Monitrol II', International Reagents Corporation, Japan), hog heart ALT (catalog No. G8225, Sigma Chemical Company, U.S.A.) and the like. Examples of lactate dehydrogenase (LDH) include chick heart LDH (catalog No. L9126, Sigma Chemical Company, U.S.A.), hog heart LDH (catalog No. L2881, Sigma Chemical Company, U.S.A.) and the like. Examples of γ-glutamyl transpeptidase (γ-GTP) include bovine kidney γ-GTP (catalog No. G4135, Sigma Chemical Company, U.S.A.), hog kidney γ-GTP (catalog No. G2262, Sigma Chemical Company, U.S.A.) and the like.

An enzyme used in the present invention can be obtained from a biological material, derived from a human body, containing the enzyme, such as serum, erythrocytes, urine or the like; from a culture of cells, derived from human, capable of producing the enzyme; or from a culture of transformed cells, to which a human gene coding for the enzyme has been integrated by gene recombination technique, capable of producing the enzyme. Examples of enzymes obtained from a biological material derived from a human body include AST and LDH each obtained from erythrocyte, and γ-GTP obtained from urine. Each of these enzymes can be obtained from the above-mentioned biological material by conventional methods. Preferred examples of cells derived from human include human hepatitis cancer cell strain BRL68 (deposited at ATCC under the accession number CL-48), human Burkitt's lymphoma cell strain Namalwa cell (deposited at ATCC under the accession number CRL-1432), human promyeloid leukemia cell strain HL-60 (deposited at ATCC under the accession number CCL-240) and the like. The above-mentioned enzymes can be obtained from cultures of these cells by conventional methods.

With respect to each of the enzymes used in the present invention, there have been a number of reports about cDNA coding for the enzyme obtained from animals or human. Therefore, it is possible to obtain the enzyme from a culture of transformed cells, to which the gene coding for the enzyme has been integrated. With respect to the cells used to obtain the culture of transformed cells, use can be made of not only the cells derived from human, but also the cells derived from an animal other than human, such as CHO cells derived from a Chinese hamster. Further, even cells of microorganisms, such as *Escherichia coli*, can be used to obtain the culture of transformed cells. In the present invention, if desired, each of the above-mentioned enzymes (including the enzymes obtained using the cells of an animal and the enzymes obtained from a biological material derived from a human body, or from a culture of cells derived from human) can be purified by using various conventional methods (such as column chromatography) in combination, before the enzyme is used to prepare the composition of the present invention.

In the present invention, when the enzyme composition contains alkaline phosphatase, an ionic magnesium (preferably, magnesium chloride) is generally added to the composition in an amount such that the composition has a magnesium concentration of from 0.05 to 30 mM, preferably from 0.1 to 5 mM for stabilizing the composition.

With respect to the aqueous medium used as component (c) of the present invention, there is no particular limitation, as long as the aqueous medium has a buffer capacity to maintain the pH of the enzyme composition at a level around neutral pH, preferably around 7 to 8. For example, as component (c), a Good's buffer solution (which can be prepared, for example, by dissolving a buffer, such as PIPES, HEPES or BES, in distilled water, followed by adjusting the pH of the resulting solution by NaOH), a phosphate buffer solution or the like can be used in a concentration of from 5 to 200 mM, preferably from 10 to 100 mM. If desired, an antiseptic agent or the like can be added to the aqueous medium.

As mentioned above, the enzyme composition of the present invention exhibits excellent storage stability, not only when the composition is stored in a frozen form, but also when the composition is stored in a liquid form under non-freeze refrigeration conditions irrespective of whether or not the composition has an experience of being frozen. That is, the advantageous effect of the composition of the present invention can be achieved irrespective of whether the composition is in a liquid form or a frozen form.

Since the enzymatic activity of the enzyme composition of the present invention is to be compared with the enzymatic activity of a biological sample, such as serum, plasma or the like, it is preferred that the physicochemical properties of the enzyme composition of the present invention, such as viscosity, specific gravity or the like, are similar to those of the biological sample to be tested. In general, when there is a difference in physicochemical properties between a biological sample (in many cases serum) and an enzyme composition, an error frequently occurs in effecting sampling by an automatic analyzer, so that accurate determination of the enzymatic activity cannot be performed ["Kensa to Gijutu (Examination and Technology)", Vol.17, No.2, 1989]. Further, it should be noted that when a stabilizer is added to an enzyme composition, the physicochemical properties of the enzyme composition actually, frequently

become much different from those of serum than those of the enzyme composition before the addition of the stabilizer.

By contrast, the physicochemical properties of the enzyme composition of the present invention, such as viscosity, specific gravity or the like, can be easily caused to approximate to those of a biological sample to be tested, e.g. serum. The viscosity and specific gravity of human serum have been reported to be in the range of from 1.07 to 1.39 cP (as measured at 37° C.) and in the range of from 1.0180 to 1.0244 (as measured at 25° C.), respectively, although they are different among individuals [see the Research Reports by HEM (human-derived enzyme materials) working groups, p.21, June, 1992]. As mentioned above, the composition of the present invention can be provided in a liquid form or a frozen form. It is preferred that the composition of the present invention has a specific gravity of from 1.015 to 1.030 at 25° C., and a viscosity of from 1.05 to 1.40 cP at 37° C. respectively, each as directly measured when the composition is in a liquid form, or as measured after thawing the composition when the composition is in a frozen form. For example, when sorbitol as a saccharide and BSA as an albumin are used in the composition of the present invention, wherein the content of BSA is 3% and the content of sorbitol is 3%, the composition has a viscosity of 1.18 cP and a specific gravity of 1.01839. When the content of BSA is 3% and the content of sorbitol is 5%, the composition has a viscosity of 1.30 cP and a specific gravity of 1.02508. These viscosity and specific gravity values fall almost within the above-mentioned ranges of the viscosity and specific gravity of human serum. The viscosity is measured under the conditions of 37° C., 50 rpm and 48 cones by means of Biorheolizer (manufactured and sold by Toki Sangyo Co., Ltd., Japan). The specific gravity is measured at 25° C. by means of Gay-Lusac pycnometer.

The enzyme composition of the present invention can be prepared, for example, at a low temperature, preferably at 2 to 8° C., by the following method. That is, the enzyme composition of the present invention can be prepared by weighing each of the components (a) to (c), dissolving each of the components (a) and (b) in component (c) (an aqueous medium) in predetermined concentrations, and adjusting the pH of the resultant solution. Further, the obtained composition may be dispensed in glass containers, such as vials, in an amount of from 1 to 10 ml per container. The dispensed composition may be used as such, or frozen at −20° C. or less by means of a freezing machine as soon as possible after the preparation thereof, and stored in the frozen state. The enzyme composition in the frozen state is used after it has been thawed spontaneously at room temperature, preferably at 2 to 25° C., and homogenized.

When the enzyme composition of the present invention is used as control serum, a reference material or a calibrator in clinical examination for the determination of an enzymatic activity, the activity of each of the enzymes contained in the composition can be determined by the same method as that for determining an enzymatic activity in a biological sample, such as serum, using reagents appropriate for determination of the enzymatic activity. Specifically, when the enzyme composition is in a liquid form, it can be used as such; and when the composition is in a frozen form, it can be used after thawing of the frozen composition. A portion of the composition dispensed in a vial is sampled into a sample cup of an automatic analyzer in an amount of, for example, 0.1 to 0.5 ml, and then the sample cup is set in the automatic analyzer. The composition of the present invention can be stored under non-freeze refrigeration conditions at, for example, 2 to 8° C. for at least 1 week after preparation. During this storage period, the activity of any of the enzymes contained in the composition is stable and undergoes no lowering. When the composition is not used immediately after preparation, the composition can be stored in the frozen state at −20° C. or less and used after being thawed. In this case, the activity of any of the enzymes contained in the composition stored in the frozen state at −20° C. or less is stable and undergoes no lowering for at least 15 months. Further, when the frozen composition is thawed and stored at 2 to 8° C., the activity of each of the enzymes can be maintained for 1 week, as in the case of the composition immediately after preparation.

That is, the enzyme composition of the present invention is a stabilized enzyme composition suitable for use in clinical examination, which can be stably stored and suffers almost no lowering of the activity of any of the enzymes contained in the composition for a relatively long period of time, namely, for at least 1 week after preparation under non-freeze refrigeration conditions at 2 to 8° C.; for at least 15 months under freezing conditions (in which the composition is in the frozen state) at −20° C. or less; and for at least 7 days under conditions for non-freeze refrigeration at 2 to 8° C. after thawing of the frozen composition. The enzyme composition of the present invention is generally used in an amount of from about 5 to about 500 μl in every measurement operation in clinical examination.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be further illustrated in more detail with reference to the following Reference Examples and Examples, which should not be construed as limiting the scope of the present invention.

The measurement of the enzymatic activity in Reference Examples and Examples was conducted at 37° C. using the following commercially available reagents. When the activity of γ-glutamyl transpeptidase (γ-GTP) was measured, the calibrator attached to the reagents was used for determining the enzymatic activity. With respect to the enzymes other than γ-GTP, the enzymatic activity was determined by a method using the calibration factor (K factor) which is obtained by the measurement using an indicator compound [see "Kensa-to-Gijutsu" (MODERN MEDICAL LABORATORY)", vol.25, No.5, p223, Extra issue in 1993]. An auto-analyzer (7070 type manufactured and sold by Hitachi, Ltd., Japan) was used for the measurement of the enzymatic activity.

Enzyme	Reagents for measurement
asparatate aminotranferase (AST)	GOTII-HA test-WAKO (manufactured and sold by WAKO PURE CHEMICAL Industries Ltd., Japan)
alanine aminotranferase (ALT)	GPTII-HA test-WAKO (manufactured and sold by WAKO PURE CHEMICAL Industries Ltd., Japan)
λ-glutamyl transpeptidase (λ-GTP)	Determiner λ-GTP (manufactured and sold by KYOWA MEDEX Co., Ltd., Japan)
alkaline phosphatase (ALP)	ALPII-HA test-WAKO (manufactured and sold by WAKO PURE CHEMICAL Industries Ltd., Japan)
creatine kinase	CPKII-HA test-WAKO

-continued

Enzyme	Reagents for measurement
(CK)	(manufactured and sold by WAKO PURE CHEMICAL Industries Ltd., Japan)
lactate dehydrogenase (LDH)	LDHII-HA test-WAKO (manufactured and sold by WAKO PURE CHEMICAL Industries Ltd., Japan)

REFERENCE EXAMPLE 1

Human promyeloid leukemia cell strain HL-60 (deposited at ATCC under the accession number CCL-240) was dispersed in a RPMI-1640 medium (manufactured and sold by Sigma Chemical Company, USA) containing fetal bovine serum added thereto in an amount of 10% (v/v), so that the resultant suspension had a cell density of 1×10^5 cells/ml. 1.5 Liters of the obtained cell suspension were charged into a 2 liter spinner flask and were subjected to suspension culture with agitation for 5 days in a carbon dioxide incubator which was set to have 37° C. and an atmosphere consisting of air (95%) and carbon dioxide (5%). The cultured cells were separated by means of a centrifuge and then, disrupted by ultrasonication. The enzymatic activity of the supernatant was measured with respect to the cell density of 10^7 cells/ml. As a result, 6435 U/liter of LDH, 260 U/liter of AST and 5300 U/liter of CK-were detected. The supernatant of the mixture obtained by the disruption of the cultured cells was subjected to ammonium sulfate fractionation, and then to column chromatographic fractionation using DEAE-Sepharose CL-6B column (manufactured and sold by Pharmacia Fine Chemicals AB, Sweden), to thereby obtain a mixed fraction of AST and LDH and a CK fraction. The mixed fraction of AST and LDH was subjected again to column chromatographic fractionation using Blue-Sepharose CL-6B column (manufactured and sold by Pharmacia Fine Chemicals AB, Sweden), so that AST was obtained from the fraction passing through the column and LDH was obtained from the fraction adsorbed on the column.

REFERENCE EXAMPLE 2

Human fetal hepatic cell strain BRL68 (deposited at ATCC under the accession number CL-48) was dispersed in a commercial MEM medium containing fetal bovine serum added thereto in an amount of 10% (v/v), so that the resultant suspension had a cell density of 2.5×10^4 cells/ml. 200 ml of the obtained cell suspension was charged into each of 225 ml flasks for tissue culture [manufactured and soled by Sumitomo Bakelite Co., Ltd., Japan] and was subjected to static culture for 4 days in a carbon dioxide incubator which was set to have 37° C. and an atmosphere consisting of air (95%) and carbon dioxide (5%). 0.01% (w/v) trypsin solution (manufactured and soled by GIBCO, USA) was added to the cultured cells so as to remove the cultured cells from the inner surface of the flask. The cells were collected by means of a centrifuge and then, disrupted by ultrasonication. The enzymatic activity of the supernatant was measured with respect to the cell density of 10^7 cells/ml, and 14650 U/liter of LDH, 811 U/liter of AST and 1473 U/ml of ALP were detected. The supernatant of the mixture obtained by the disruption of the cultured cells was subjected to ammonium sulfate fractionation, and then to column chromatographic fractionation using Blue-Sepharose CL6B column (manufactured and sold by Pharmacia Fine Chemicals AB, Sweden). ALP was obtained from the fraction passing through the column.

EXAMPLE 1

Enzyme compositions having the following composition were provided:

20 mM PIPES-NaOH (pH 7.5),

3% BSA (bovine serum albumin) (manufactured and sold by Sigma Chemical Company, USA),

500 U/liter ALP (hog kidney-derived; manufactured and sold by Sigma Chemical Company, USA), and

250 U/liter CK (rabbit muscle-derived; Boehringer-Mannheim GmbH, Germany).

From the enzyme compositions, the following samples were prepared:

(1) a sample having the above composition, which contains ALP and CK,

(2) a sample which was prepared by adding 1 mM of N-acetylcysteine (manufactured and sold by Sigma Chemical Company, USA) to sample (1) above,

(3) a sample which was prepared by adding 0.5 mM of magnesium chloride to sample (1) above,

(4) a sample which was prepared by adding 5% sorbitol (manufactured and sold by WAKO PURE CHEMICAL Industries, Japan) to sample (1) above, and

(5) a sample which was prepared by adding 0.5 mM of magnesium chloride and 3% sorbitol to sample (1) above.

The residual enzymatic activity after the storage at 37° C. for one day was measured with respect to each of samples (1) to (5). The respective residual activities (%) of ALP and CK are shown in Table 1 with respect to each sample.

TABLE 1

Residual activity (%) after storage at 37° C. for one day

	Additives other than BSA	Enzyme	Residual activity (%)
Controls	(1)	ALP	65
	None	CK	90
	(2)	ALP	34
	N-acetylcysteine	CK	98
	(3)	ALP	75
	$MgCl_2$	CK	63
Present invention	(4)	ALP	98
	Sorbitol	CK	99
	(5)	ALP	100
	Sorbitol $MgCl_2$	CK	99

As shown in Table 1, with respect to sample (1) in which only BSA was added to a buffer solution of enzymes, the respective residual activities of ALP and CK were caused to lower as compared to the original activities. With respect to sample (2) in which N-acetylcysteine was added, the residual activity of ALP was caused to lower even as compared to that in sample (1), although the residual activity of CK was a little improved as compared to that in sample (1). With respect to sample (3) which had magnesium chloride added thereto, contrary to the case of sample (2), the residual activity of ALP was a little improved as compared to that in sample (1), whereas the residual activity of CK was caused to lower as compared to that in the sample (1). By contrast, with respect to samples (4) and (5), both of which are the stabilized enzyme compositions of the present

invention, almost no lowering of the activity of each of ALP and CK was observed.

EXAMPLE 2

Enzyme compositions having the following composition were provided:

20 mM BES-NaOH (pH 7.5),

3% BSA (manufactured and sold by Sigma Chemical Company, USA),

0.5 mM magnesium chloride,

2 mM alanine,

526 U/liter ALP (hog kidney-derived; manufactured and sold by Sigma Chemical Company, USA),

303 U/liter CK (rabbit muscle-derived; Boehringer-Mannheim GmbH, Germany), and

108 U/liter ALT (hog heart-derived; manufactured and sold by Sigma Chemical Company, USA).

To the above-provided enzyme compositions were respectively added trehalose, sorbitol, mannitol, galactose and lactose, each in a concentration of 5%.

With respect to each of the resultant samples containing a saccharide, the storage stability in a liquid form was examined after the storage at 5° C. and the storage stability in a frozen form was examined after the storage at −20° C.

The respective residual activities (%) of the enzymes after the storage at 5° C. for 3 weeks are shown in Table 2. The respective residual activities (%) of the enzymes after the storage at −20° C. for 3 weeks are shown in Table 3.

TABLE 2

Residual activity (%) after storage at 5° C. for 3 weeks

	Saccharide	ALT	ALP	CK
Controls	None	95	99	94
	Mannitol	95	98	94
	Galactose	90	103	95
	Lactose	87	109	95
Present invention	Trehalose	98	99	100
	Sorbitol	99	99	98

TABLE 3

Residual activity (%) after storage at −20° C. for 3 weeks

	Saccharide	ALT	ALP	CK
Controls	None	97	96	95
	Mannitol	82	94	92
	Galactose	100	104	99
	Lactose	100	103	101
Present invention	Trehalose	101	99	98
	Sorbitol	100	98	98

As shown in Table 2, when the samples were stored at 5° C. for 3 weeks, with respect to the residual activity of each of the samples respectively having galactose and lactose added thereto, the activity of ALT was caused to lower by approximately 10% as compared to the original activity. With respect to the residual activity of each of the sample containing no saccharide and the sample having mannitol added thereto, the activity of ALT was caused to lower by approximately 5% as compared to the original activity. The activity of ALP was maintained at substantially the same level as the original level of activity, with respect to all of the samples (including the sample containing no saccharide). The activity of CK was caused to lower by approximately 5% with respect to the samples falling outside the scope of the present invention.

As shown in Table 3, when the samples were stored at −20° C. for 3 weeks, with respect to the residual activity of the sample containing no saccharide, the activity of CK was caused to lower by approximately 5% as compared to the original activity. With respect to the sample having mannitol added thereto, the activity of ALT was caused to lower by approximately 18%, the activity of ALP was caused to lower by approximately 6% and the activity of CK was caused to lower by approximately 8%, as compared to the respective original activities.

By contrast, with respect to the samples respectively having sorbitol and trehalose added thereto, both of which are the stabilized enzyme compositions of the present invention, almost no lowering of the activity of each of the enzymes contained therein was observed under both storage conditions of 5° C. and −20° C.

EXAMPLE 3

Enzyme compositions having the following composition were provided:

20 mM PIPES-NaOH (pH 7.5),

0.3% BSA (manufactured and sold by Sigma Chemical Company, USA),

0.5 mM magnesium chloride,

0.5 mM calcium chloride,

10 mM sodium glutamate

536 U/liter ALP (hog kidney-derived; manufactured and sold by Sigma Chemical Company, USA),

301 U/liter CK (rabbit muscle-derived; Boehringer-Mannheim GmbH, Germany),

100 U/liter AST (hog heart-derived; manufactured and sold by Sigma Chemical Company, USA),and

108 U/liter ALT (hog heart-derived; manufactured and sold by Sigma Chemical Company, USA).

From the above enzyme compositions were prepared three samples, namely, a sample having 5% sucrose added thereto, a sample having 5% trehalose added thereto, and a sample having no saccharide added thereto.

Each of the samples was stored at −20° C. in a frozen form. The respective residual activities (%) of the samples after the storage at −20° C. for 6 months are shown in Table 4.

TABLE 4

Residual activity (%) after storage at −20° C. for 6 months

	Saccharide	AST	ALP	CK	ALT
Controls	None	101	92	100	99
	Sucrose	99	85	99	97
Present invention	Trehalose	100	100	100	99

As shown in Table 4, when the samples were stored at −20° C. for 6 months, with respect to the sample having sucrose added thereto, the activity of ALP was caused to lower by approximately 15% as compared to the original activity. With respect to the sample containing no saccharide, the activity of ALP was caused to lower by approximately 10% as compared to the original activity. By contrast, with respect to the sample having trehalose added thereto, which is the stabilized enzyme composition of the present invention, almost no lowering of the activity of each of AST, ALT, ALP and CK was observed.

EXAMPLE 4

Enzyme compositions having the following composition were provided:

20 mM BES-NaOH (pH 7.5),

3% BSA (manufactured and sold by Sigma Chemical Company, USA),

2 mM magnesium chloride,

0.05% sodium azide,

109 U/liter ALP (obtained from human fetal hepatic cell strain BRL 68 by the same method as in the Reference Example 2),

61 U/liter CK (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1),

38 U/liter AST (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1),

34 U/liter ALT (hog heart-derived; manufactured and sold by Sigma Chemical Company, USA),

31 U/liter γ-GTP (bovine kidney-derived; manufactured and sold by Sigma Chemical Company, USA), and

103 U/liter LDH (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1).

From the above enzyme compositions, the following samples were prepared:

(1) a sample which was prepared by adding trehalose to the above enzyme composition so that the sample had a trehalose concentration of 0.5%,

(2) a sample which was prepared by adding trehalose to the above enzyme composition so that the sample had a trehalose concentration of 2%,

(3) a sample which was prepared by adding trehalose to the above enzyme composition so that the sample had a trehalose concentration of 5%.

The residual enzymatic activity after the storage at 5° C. and −20° C. for 7 days was measured with respect to each of samples (1) to (3) to examine the influence of the concentration of trehalose on the enzymatic activity. The respective residual activities (%) of the enzymes after the storage at −20° C. for 7 days are shown in Table 5 and the respective residual activities (%) of the enzymes after the storage at 5° C. for 7 days are shown in Table 6.

TABLE 5

Residual activity (%) after storage at −20° C. for 7 days

		AST	ALT	γ-GTP	ALP	CK	LDH
Control	0.5% Trehalose	97	96	100	98	101	101
Present invention	2% Trehalose	100	100	101	100	99	100
	5% Trehalose	100	99	101	100	100	100

TABLE 6

Residual activity (%) after storage at 5° C. for 7 da s

		AST	ALT	γ-GTP	ALP	CK	LDH
Control	0.5% Trehalose	95	102	99	98	98	90

TABLE 6-continued

Residual activity (%) after storage at 5° C. for 7 days

		AST	ALT	γ-GTP	ALP	CK	LDH
Present invention	2% Trehalose	101	99	100	99	99	99
	5% Trehalose	102	98	100	98	99	101

As shown in Table 5, when the samples were stored at −20° C. for 7 days, any one of the enzyme components showed almost no lowering of the activity except that, with respect to sample (1) which had 0.5% trehalose added thereto, the activity of ALT was caused to a little lower as compared to the original activity. Whereas, as shown in Table 6, when the samples were stored at 5° C. for 7 days, the activity of LDH was caused to lower by 10% with respect to the sample (1) which had 0.5% trehalose added thereto, indicating that the above enzyme composition was not stabilized at 5° C. by adding trehalose in an amount of 0.5% or so. In this connection, it is to be noted that, in the case of the storage at 25° C. for 7 days, the activity of LDH was maintained at the original level at trehalose concentrations of 0.5%, 2% and 5%. It is suggested that LDH is unstable at lower temperatures. This inactivation of LDH at lower temperatures was not observed with respect to the samples respectively having 2% and 5% trehalose added thereto. The above shows that 2% or more trehalose is required to stabilize the above enzyme compositions.

EXAMPLE 5

Enzyme compositions of the following composition were provided:

20 mM PIPES-NaOH (pH 7.5),

3% BSA (manufactured and sold by Sigma Chemical Company, USA),

2 mM magnesium chloride,

0.05% sodium azide,

462 U/liter ALP (obtained from human fetal hepatic cell strain BRL 68 by the same method as in the Reference Example 2),

282 U/liter CK (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1),

100 U/liter AST (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1),

118 U/liter ALT (hog heart-derived; manufactured and sold by Sigma Chemical Company, USA),

146 U/liter γ-GTP (bovine kidney-derived; manufactured and sold by Sigma Chemical Company, USA), and

235 U/liter LDH (obtained from human promyeloid leukemia cell strain HL-60 by the same method as in the Reference Example 1).

From the above enzyme compositions, the following samples were prepared:

To the above-provided enzyme compositions were respectively added sorbitol, trehalose, mannitol and sucrose, each in a concentration of 5%.

The storage stability in a frozen form was examined after the storage at −20° C. The respective residual activities (t) of

the enzymes after the storage at −20° C. for 9 months are shown in Table 7.

TABLE 7

Residual activity (%) after storage at −20° C. for 9 months

	Controls		Present invention		
	None	Mannitol	Sucrose	Sorbitol	Trehalose
AST	92	98	98	101	102
ALT	66	90	96	100	102
λ-GTP	101	102	98	101	101
ALP	87	98	76	102	102
CK	95	91	97	100	100
LDH	94	101	97	102	102

As shown in Table 7, when the samples were stored at −20° C. for 9 months, with respect to the samples containing no saccharide, the respective activities of AST, ALT, ALP, CK and LDH were caused to lower by 8 to 34% as compared to the original activities. With respect to the samples having mannitol added thereto, the activities of ALT and CK were caused to lower by approximately 10% as compared to the original activities. With respect to the samples having sucrose added thereto, the respective activities of ALT, ALP and CK were caused to lower by 4 to 24% as compared to the original activities. By contrast, with respect to the samples respectively having sorbitol and trehalose added thereto, both of which are the stabilized enzyme compositions of the present invention, almost no lowering of the activity of each of the enzymes contained therein was observed.

Further, with respect to the above-mentioned samples respectively having sorbitol and trehalose added thereto, comparison was made between the residual activities of enzymes of a sample stored at 5° C. for 1 week after preparation thereof and those of a sample which was stored in a frozen form at −20° C. for 9 months and then subjected to thawing, followed by storage at 5° C. for 1 week after the thawing. The results are shown in Table 8.

TABLE 8

Residual activity (%) after storage at 5° C. for 7 da s

	A sample immediately after preparation	A sample thawed after the storage in a frozen form at −20° C. for 9 months	A sample immediately after preparation	A sample thawed after the storage in a frozen form at −20° C. for 9 months
AST	100	99	100	100
ALT	99	99	99	99
λ-GTP	100	99	101	100
ALP	101	101	102	99
CK	99	98	99	100
LDH	100	101	101	100

As shown in Table 8, almost no difference in the residual activity was observed between the samples. immediately after preparation and the samples thawed after freezing. The samples which were thawed after the storage at −20° C. for 9 months were stable upon storage at 5° C. for 1 week, similarly to the samples immediately after preparation.

INDUSTRIAL APPLICABILITY

The enzyme composition of the present invention-comprising an enzyme component comprising at least two enzymes selected from the group consisting of alkaline phosphatase, creatine kinase and alanine aminotransferase; and a stabilizer component comprising effective stabilizing amounts of an albumin, and at least one saccharide selected from the group consisting of trehalose and sorbitol, shows almost no lowering of enzymatic activity, with respect to all enzymes contained therein, for at least one week when it is stored at 2 to 8° C. after preparation, for at least 15 months when it is frozen at −20° C. or lower, and for at least one week even when it is stored at 2 to 8° C. after thawing. This indicates that the enzyme composition of the present invention has extremely excellent storage stability. Therefore, the enzyme composition of the present invention can be advantageously used for the purpose of checking the precision in measurement, correcting measured values and calibrating the amount and activity of an enzyme, in a clinical examination for measuring the enzymatic activity in a sample, such as serum or the like.

What is claimed is:

1. A stabilized enzyme composition for use as a clinical diagnostic reagent, comprising:

(a) an enzyme component consisting essentially of a multiplicity of enzymes, said enzymes comprising alkaline phosphatase creatine kinase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and γ-glutamyl transpeptidase;

(b) a stabilizer component in an amount sufficient to stabilize said enzyme component (a), wherein said stabilizer component comprises an albumin and sorbitol, wherein said sorbitol is at a concentration of 2–5% (w/v); and

(c) an aqueous medium having dissolved therein said components (a) and (b).

2. The composition according to claim **1**, wherein said alkaline phosphatase is present in a concentration of from 9 to 6500 U, said creatine kinase is present in a concentration of from 6 to 4000 U, said alanine aminotransferase is present in a concentration of from 3 to 1150 U, said aspartate aminotransferase is present in a concentration of from 3 to 1150 U, said lactate dehydrogenase is present in a concentration of from 8 to 4000 U, and said γ-glutamyl transpeptidase is present in a concentration of from 2 to 1200 U each per liter of said composition.

3. The composition according to claim **1**, wherein, with respect to said stabilizer component (b), said albumin is present in a concentration of from 0.3 to 7 (w/v) %, based on the volume of said composition.

4. The composition according to claim **1**, which is in a liquid form or in a frozen form.

5. The composition according to claim **1**, wherein the enzymes of said enzyme component (a) are obtained from biological materials, derived from a human body, containing the enzymes; from cultures of cells, derived from human, capable of producing the enzymes; or from cultures of transformed cells, into which human genes coding for the enzymes have been integrated by gene recombination technique, capable of producing the enzymes.

6. The composition according to claim **5**, which has a specific gravity of from 1.015 to 1.030 at 25° C., and a viscosity of from 1.05 to 1.40 cP at 37° C., each as directly measured when said composition is in a liquid form, or as measured after thawing said composition when said composition is in a frozen form.

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US006969600B2

(12) **United States Patent**
Kimoto et al.

(10) **Patent No.: US 6,969,600 B2**
(45) **Date of Patent: *Nov. 29, 2005**

(54) **CARBONYL REDUCTASE, METHOD FOR PRODUCING SAID ENZYME, DNA ENCODING SAID ENZYME, AND METHOD FOR PRODUCING ALCOHOL USING SAID ENZYME**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 831 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/855,309**

(22) Filed: **May 14, 2001**

(65) **Prior Publication Data**

US 2004/0197773 A1 Oct. 7, 2004

Related U.S. Application Data

(62) Division of application No. 09/468,738, filed on Dec. 21, 1999, now Pat. No. 6,312,933.

(30) **Foreign Application Priority Data**

Dec. 21, 1998 (JP) 10-363130
Jun. 17, 1999 (JP) 11-171160

(51) **Int. Cl.**[7] **C12N 9/02**; C12Q 1/26; C12P 21/06; C07H 21/04

(52) **U.S. Cl.** **435/189**; 435/25; 435/69.1; 435/252.3; 536/23.2

(58) **Field of Search** 435/189, 25, 69.1, 435/190, 440; 536/23.2

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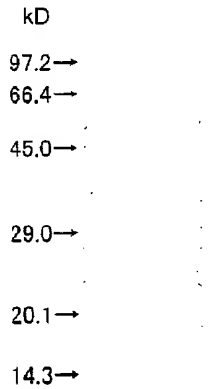
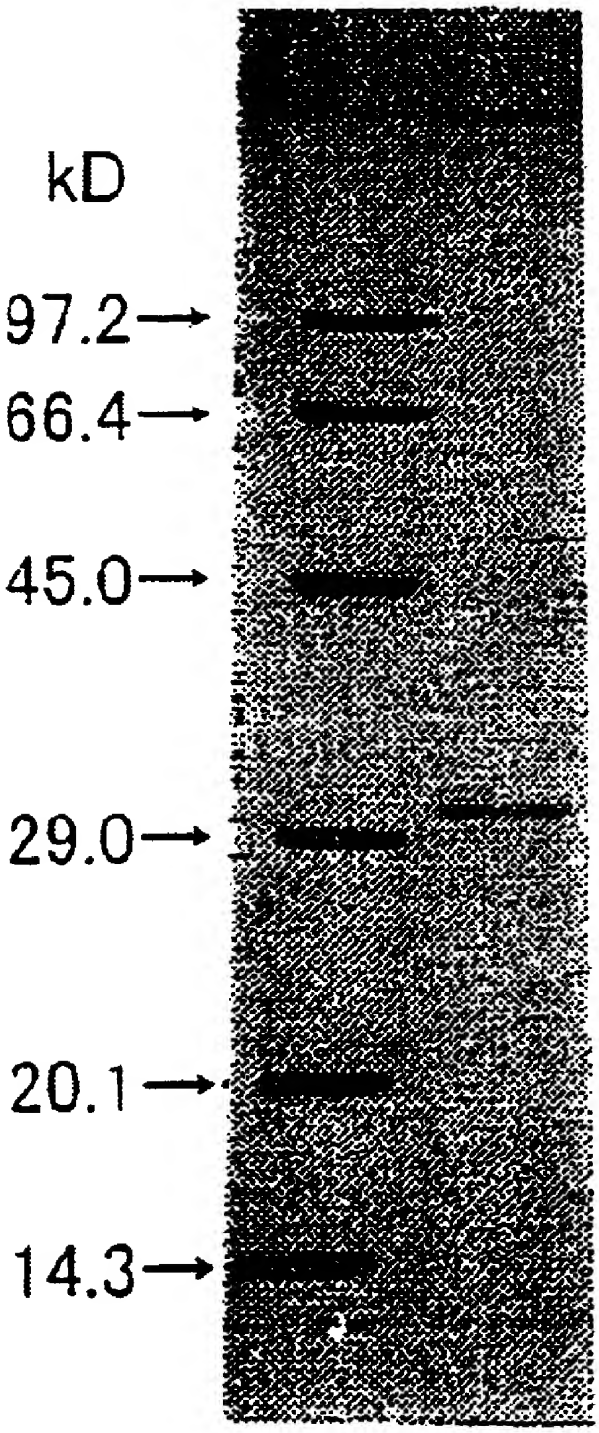
(57) **ABSTRACT**

A novel carbonyl reductase useful for producing alcohol, particularly derivatives of (S)-4-halo-3-hydroxybutyrate ester, is provided. A novel carbonyl reductase derived from *Kluyveromyces aestuarii* and the nucleic acid encoding the enzyme are provided. The carbonyl reductase has excellent reductase activity and stereoselectivity. The carbonyl reductase reduces ketone to produce alcohol. It can be particularly advantageous when used in industrial production of (S)-4-halo-3-hydroxybutyrate ester.

1 Claim, 5 Drawing Sheets





FI . 1

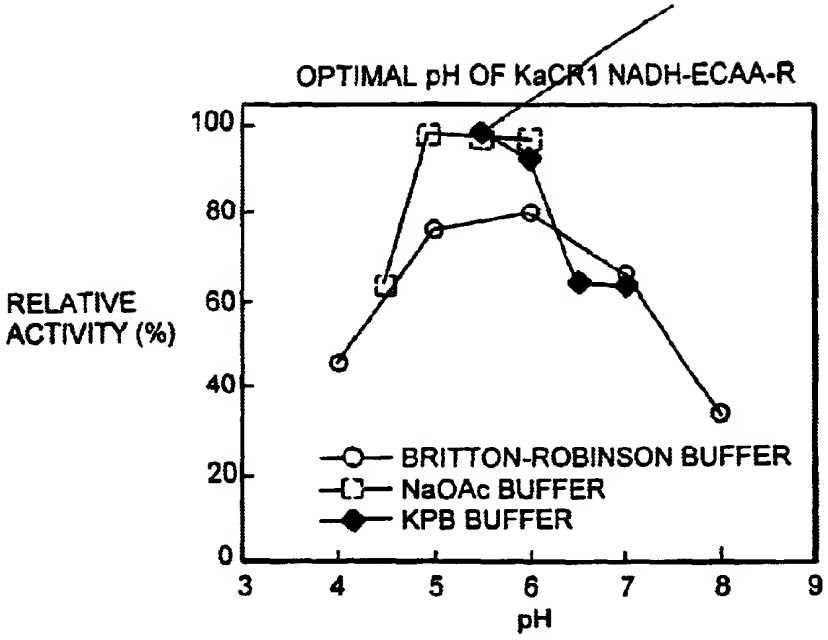

OPTIMAL pH OF KaCR1 NADH-ECAA-R
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
3
4
5
6
7
8
9
pH
BRITTON-ROBINSON BUFFER
NaOAc BUFFER
KPB BUFFER

FIG. 2

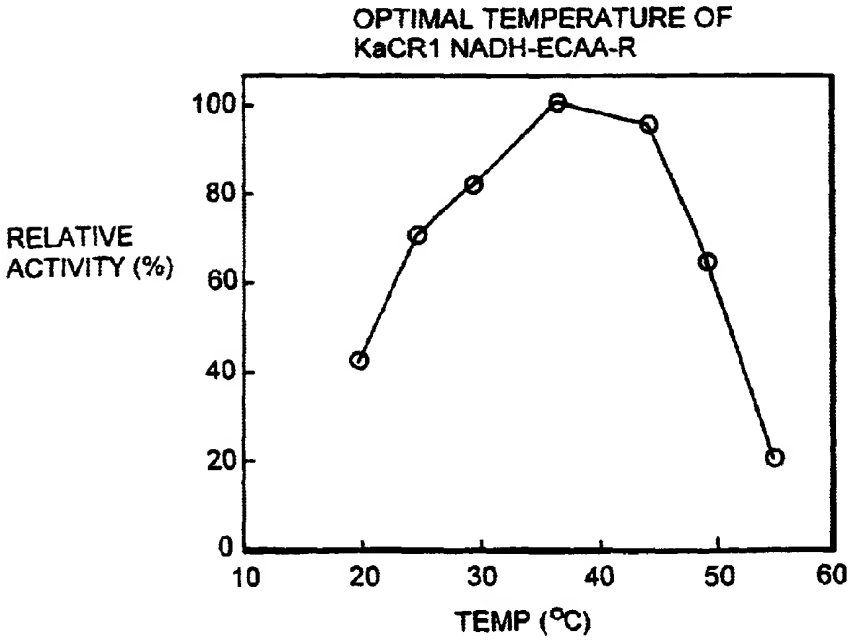

OPTIMAL TEMPERATURE OF KaCR1 NADH-ECAA-R
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
10
20
30
40
50
60
TEMP (°C)

FIG. 3

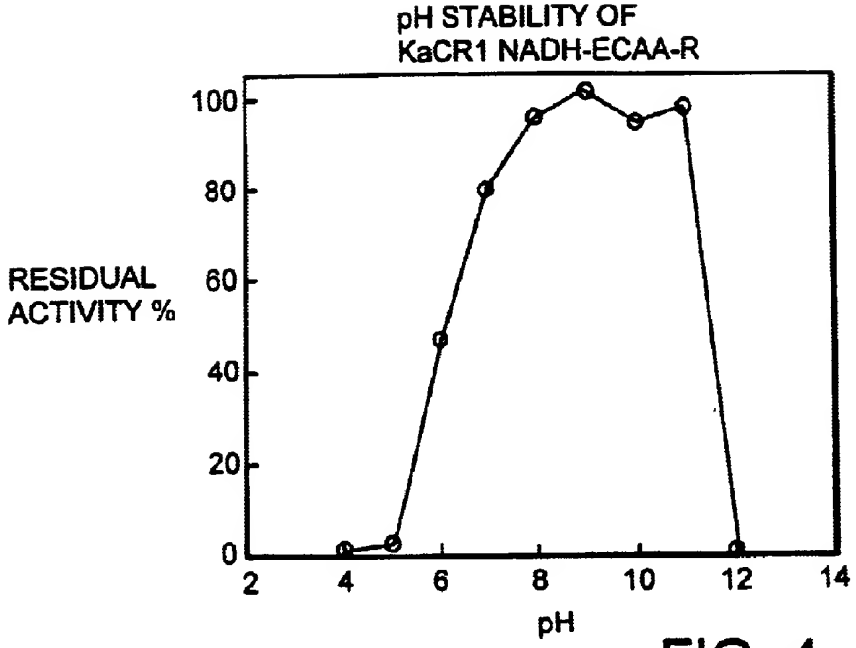

pH STABILITY OF
KaCR1 NADH-ECAA-R
RESIDUAL
ACTIVITY %
100
80
60
40
20
0
2
4
6
8
10
12
14
pH

FIG. 4

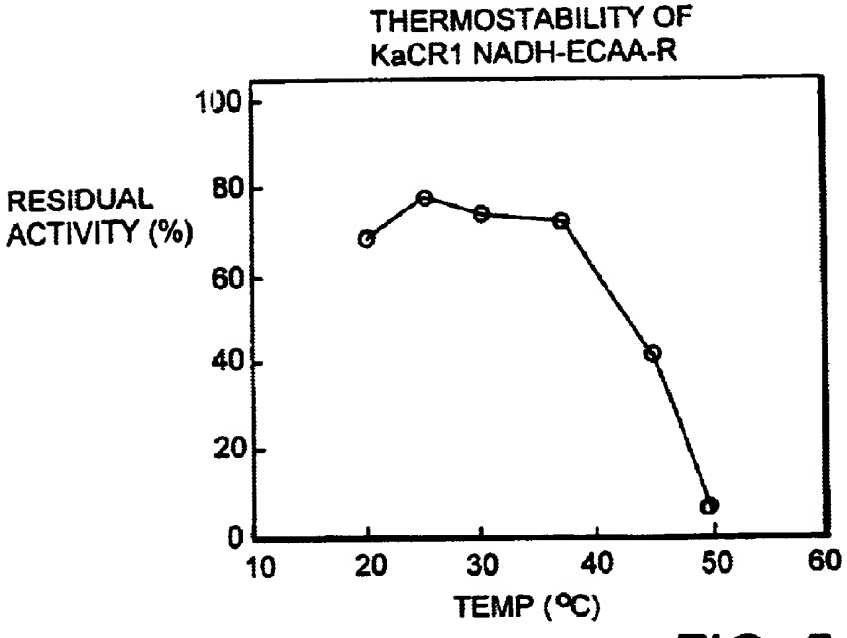

THERMOSTABILITY OF
KaCR1 NADH-ECAA-R
RESIDUAL
ACTIVITY (%)
100
80
60
40
20
0
10
20
30
40
50
60
TEMP (°C)

FIG. 5

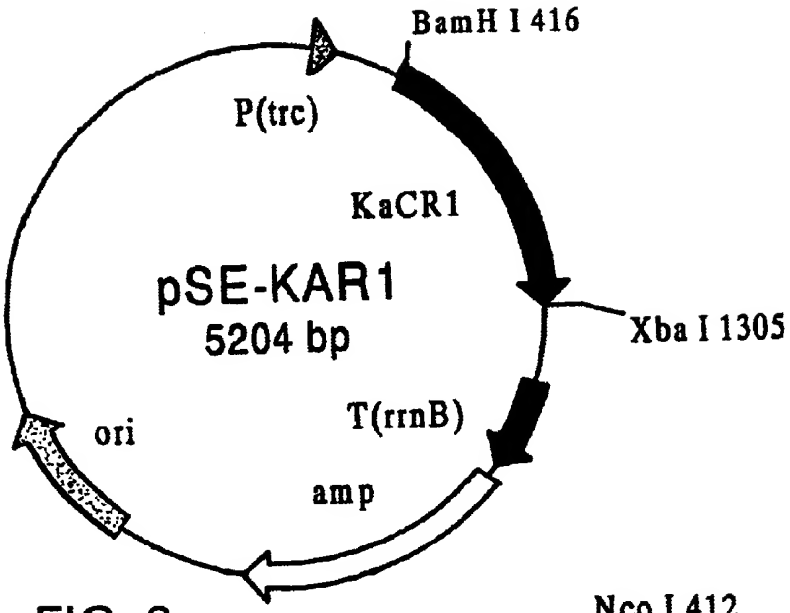

BamH I 416
P(trc)
KaCR1
pSE-KAR1
5204 bp
Xba I 1305
T(rrnB)
ori
amp

FIG. 6

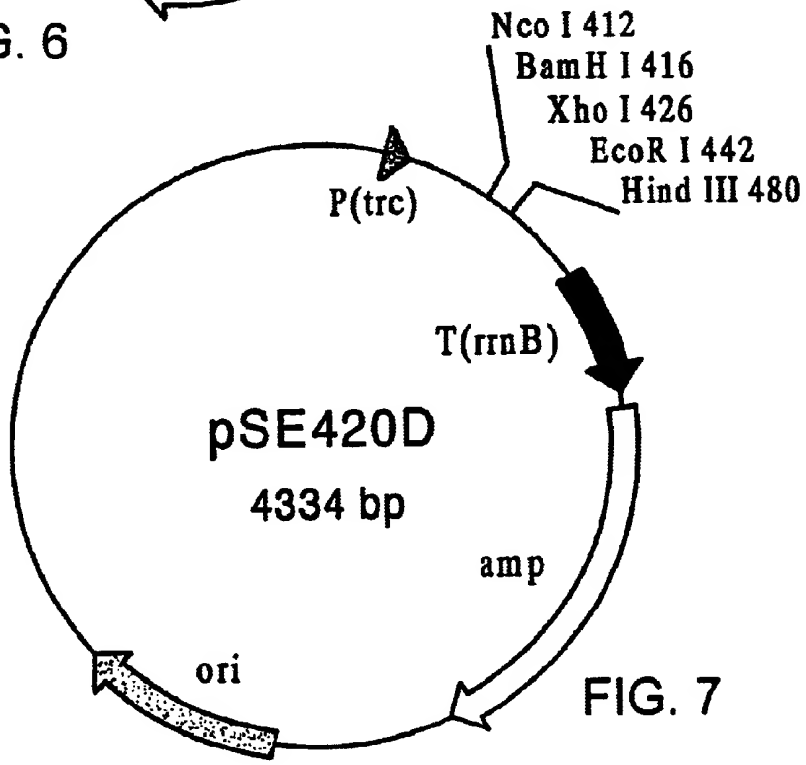

Nco I 412
BamH I 416
Xho I 426
EcoR I 442
Hind III 480
P(trc)
T(rrnB)
pSE420D
4334 bp
amp
ori

FIG. 7

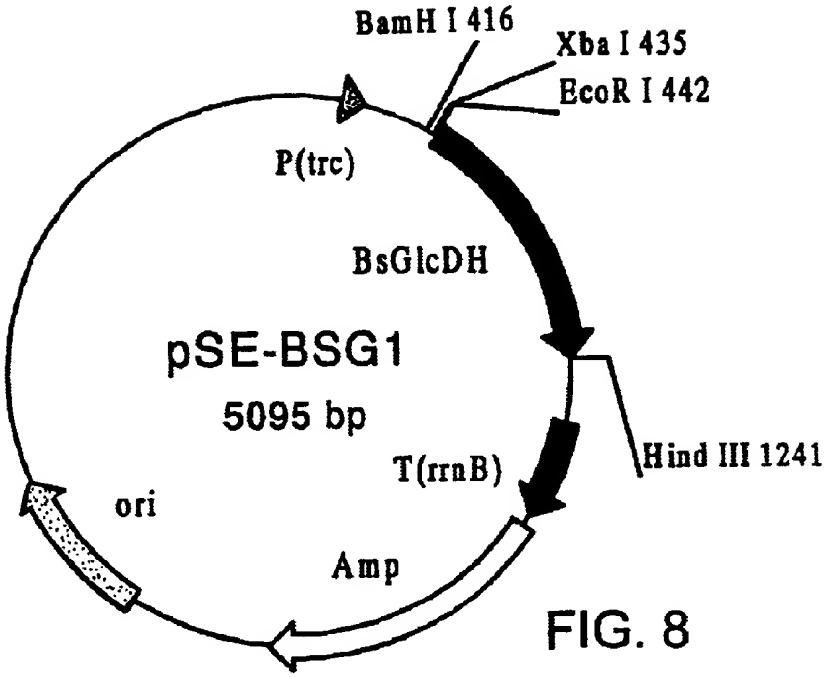

BamH I 416
Xba I 435
EcoR I 442
P(trc)
BsGlcDH
pSE-BSG1
5095 bp
T(rrnB)
Hind III 1241
ori
Amp

FIG. 8

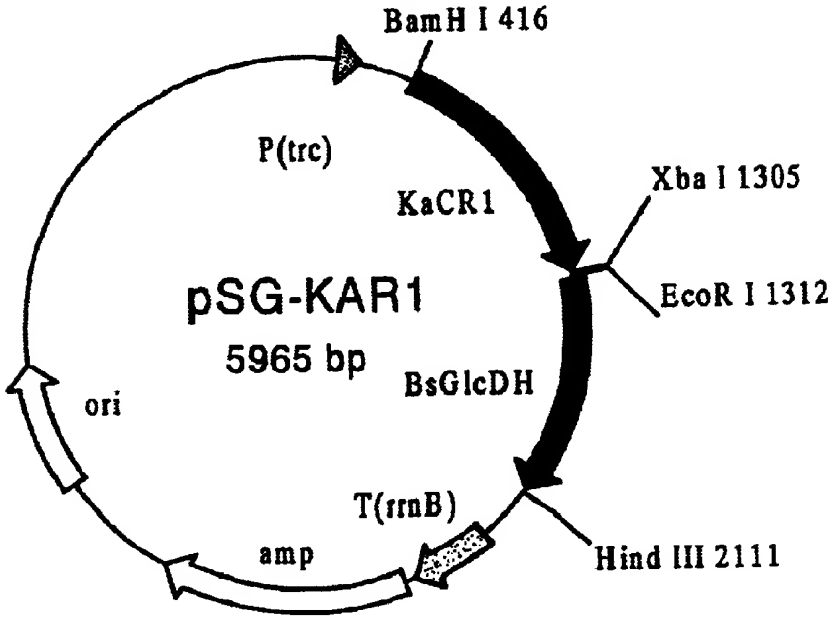

BamH I 416
P(trc)
KaCR1
Xba I 1305
pSG-KAR1
5965 bp
EcoR I 1312
BsGlcDH
ori
T(rrnB)
amp
Hind III 2111

FIG. 9

CARBONYL REDUCTASE, METHOD FOR PRODUCING SAID ENZYME, DNA ENCODING SAID ENZYME, AND METHOD FOR PRODUCING ALCOHOL USING SAID ENZYME

This application is a divisional of U.S. patent application Ser. No. 09/468,738, filed Dec. 21, 1999 now issued as U.S. Pat. No. 6,312,933, which claims priority from Japanese Patent Application Nos. 10/363130, filed Dec. 21, 1998, and 11/171160, filed Jun. 17, 1999.

FIELD OF INVENTION

The present invention relates to a novel, reduced beta-nicotinamide adenine dinucleotide-dependent carbonyl reductase that is useful for producing alcohol, particularly (S)-4-halo-3-hydroxybutyrate ester; DNA encoding said enzyme; a method for producing said enzyme; and a method for producing alcohol, particularly (S)-4-halo-3-hydroxybutyrate ester, using said enzyme.

BACKGROUND OF THE INVENTION

Asymmetric reduction methods using microorganisms such as baker's yeast to produce optically active (S)-4-halo-3-hydroxybutyrate ester (Unexamined Published Japanese Patent Application No. (JP-A) Sho 61-146191, JP-A Hei 6-209782, and so on) have been known for some time. These production methods, however, have problems that must be solved for industrial applications because the optical purity and yield of the product are low due to more than one reductases existing in microbial cells. Optically active (S)-4-halo-3-hydroxybutyrate ester is utilized as a synthetic intermediate of drugs. It is thus important in the chemical industry to determine how to produce (synthesize or resolve) optically purified antipodes of the compound.

Enzymes capable of producing (S)-4-halo-3-hydroxybutyrate ester from 4-haloacetoacetate ester are described below, and methods for synthesizing (S)-4-halo-3-hydroxybutyrate ester using these enzymes have been reported.

3-Hydroxysteroid dehydrogenase (JP-A Hei 1-277494)

Glycerol dehydrogenase (Tetrahedron Lett. 29, 2453–2454 (1988))

Alcohol dehydrogenase derived from *Pseudomonas* sp. PED (J. Org. Chem. 57, 1526–1532 (1992))

Reductases derived from baker's yeast (D-enzyme-1, D-enzyme-2, J. Am. Chem. Soc. 107, 2993–2994 (1985))

Aldehyde dehydrogenase 2 derived from *Sporobolomyces salmonicolor* (Abstract of 391st Meeting of the Kansai Branch of the Japan Society of Bioscience, Biotechnology, and Agrochemistry, p37 (1995))

Ketopantothenate reductase derived from *Candida macedoniensis* (Arch. Biochem. Biophys. 294, 469–474 (1992))

Ethyl 4-chloroacetoacetate reductase derived from *Geotrichum candidum* (Enzyme Microb. Technol. 14, 731–738 (1992))

Carbonyl reductase derived from *Candida magnoliae* (WO98/35025)

Carbonyl reductase derived from *Kluyveromyces lactis* (JP-A Hei 11-187869)

Most of these enzymes are reductases that require reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme. Thus, the method for synthesizing (S)-4-halo-3-hydroxybutyrate ester using these enzymes is industrially disadvantageous because it needs the addition and regeneration of expensive and chemically unstable NADPH.

3-Hydroxysteroid dehydrogenase, glycerol dehydrogenase, and alcohol dehydrogenase derived from Pseudomonas sp. PED are oxidoreductases, which catalyze not only reduction reactions using reduced nicotinamide adenine dinucleotide (NADH) as an electron donor but also oxidation (dehydrogenation) reactions. The use of these enzymes cannot produce (S)-4-halo-3-hydroxybutyrate ester in a high yield because the equilibrium of the enzymatic reaction is likely to limit the reaction rate.

SUMMARY OF THE INVENTION

An objective of this invention is to provide a carbonyl reductase that uses NADH as a coenzyme. Another objective of this invention is to provide a carbonyl reductase with excellent stereoselectivity capable of acting on a substrate, 4-haloacetoaectate ester, to produce optically active (S)-4-halo-3-hydroxybutyrate ester with high optical purity at a high yield.

Still another objective of the present invention is to isolate a DNA encoding the carbonyl reductase with desired properties and obtaining a recombinant enzyme. A further objective of the invention is to provide a method for enzymatically producing optically active (S)-4-halo-3-hydroxybutyrate ester using a novel carbonyl reductase.

Another objective of the invention is to obtain a recombinant capable of simultaneously expressing not only the desired enzyme described above but also an enzyme that reduces NAD^+ to NADH. It is also an objective of the invention to provide a method for enzymatically producing optically active (S)-4-halo-3-hydroxybutyrate ester using a novel carbonyl reductase, involving an enhanced regeneration system of the coenzyme using the above recombinant.

The present inventors thought that carbonyl reductases capable of utilizing NADH as an electron donor would be industrially useful since NADH is less expensive and chemically more stable than NADPH. We also thought that enzymes that reduce 4-haloacetoacetate ester to form (S)-4-halo-3-hydroxybutyrate ester but do not substantially dehydrogenate the formed (S)-4-halo-3-hydroxybutyrate ester would be useful for efficiently producing optically active (S)-4-halo-3-hydroxybutyrate ester.

The present inventors sought enzymes that meet the above requirements and found a desired enzyme from *Kluyveromyces aestuarii*.

We succeeded in isolating a novel enzyme and DNA encoding the enzyme, and in developing a method for producing alcohol using this enzyme. The present invention relates to the carbonyl reductase described below, DNA encoding said enzyme, a method for producing said enzyme, and the use of said enzyme.

1. A carbonyl reductase having the following physicochemical properties:

Reactivity

It reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor.

Substrate Specificity

It has high reductase activity for 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester and

shows higher enzymatic activity when used with reduced beta-nicotinamide adenine dinucleotide as an electron donor than reduced beta-nicotinamide adenine dinucleotide phosphate.

2. The carbonyl reductase described in 1, which has additional physicochemical properties below:

Optimal pH

5.0 to 6.0

Substrate Specificity

It does not substantially dehydrogenate isopropanol and does not reduce acetoacetate.

Molecular Weight

About 32,000 when determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis.

3. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2 and having the enzymatic activity for catalyzing the reduction of 4-haloacetoacetate ester to (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor.

4. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2 containing up to 30 conservative amino acid substitutions, and having the following enzymatic activities:

reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor;

has high reductase activity for 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxy-butyrate ester; and

shows higher enzymatic activity when used with reduced beta-nicotinamide adenine dinucleotide as an electron donor than reduced beta-nicotinamide adenine dinucleotide phosphate.

5. A substantially pure polypeptide encoded by a nucleic acid that hybridizes with the nucleic acid consisting the nucleotide sequence represented by SEQ ID NO: 1 under stringent conditions, and having the following enzymatic activities:

reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor;

has high reductase activity for 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester; and

shows higher enzymatic activity when used with reduced beta-nicotinamide adenine dinucleotide as an electron donor than reduced beta-nicotinamide adenine dinucleotide phosphate.

6. The substantially pure polypeptide described in 5, comprising an amino acid sequence having at least 70% homology with the amino acid sequence represented by SEQ ID NO: 2.

7. An isolated nucleic acid encoding the polypeptide described in 3.

8. An isolated nucleic acid encoding the polypeptide described in 4.

9. An isolated nucleic acid encoding the polypeptide described in 5.

10. An isolated nucleic acid encoding the polypeptide described in 3 comprising the nucleotide sequence represented by SEQ ID NO: 1.

11. An isolated nucleic acid hybridizing with the nucleic acid consisting of the nucleotide sequence represented by SEQ ID NO: 1 under stringent conditions, and encoding a polypeptide having the following enzymatic activities:

reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor;

has high reductase activity for 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester; and

shows higher enzymatic activity when used with reduced beta-nicotinamide adenine dinucleotide as an electron donor than reduced beta-nicotinamide adenine dinucleotide phosphate.

12. The nucleic acid of claim 11 comprising a nucleotide sequence having at least 70% homology with the nucleotide sequence represented by SEQ ID NO: 1.

13. A recombinant vector comprising the nucleic acid described in 7.

14. A recombinant vector comprising the nucleic acid described in 8.

15. A recombinant vector comprising the nucleic acid described in 9.

16. A transformant carrying the vector described in 13.

17. A transformant carrying the vector described in 14.

18. A transformant carrying the vector described in 15.

19. The transformant described in 16, which is a microorganism.

20. A method for producing a carbonyl reductase, the method comprising culturing the transformant described in 16.

21. A recombinant vector comprising the nucleic acid described in 7 and the nucleic acid encoding a glucose dehydrogenase.

22. The vector described in 21, wherein a glucose dehydrogenase is derived from *Bacillus subtilis*.

23. A transformant carrying the vector described in 21.

24. The transformant described in 21, which is a microorganism.

25. A method for producing the enzyme described in 1, the method comprising culturing a microorganism belonging to the genus *Kluyveromyces* and producing the enzyme described in 1.

26. The method for producing the enzyme described in 25, wherein the enzyme comprises the amino acid sequence represented by SEQ ID NO: 2.

27. The method for producing the enzyme described in 25, wherein the microorganism belonging to the genus *Kluyveromyces* is *Kluyveromyces aestuarii*.

28. A method for producing a polypeptide encoded by the nucleic acid described in 7, the method comprising culturing the transformant described in 16.

29. The method for producing the polypeptide described in 28, wherein the transformant is a microorganism.

30. A method for producing alcohol, the method comprising reacting ketone with the carbonyl reductase described in 1, microorganisms producing it, or treated microorganisms.

31. The method for producing alcohol, wherein the carbonyl reductase comprises the amino acid sequence represented by SEQ ID NO: 2.

32. The method for producing alcohol described in 30, wherein the microorganism is the transformant described in 16.

33. The method for producing alcohol described in 30, wherein ketone is a derivative of 4-haloactoacetate ester, and alcohol is a derivative of (S)-4-halo-3-hydroxybutyrate ester.

34. The method for producing alcohol described in 33, wherein the derivative of ethyl 4-haloactoacetate is 4-chloroacetoacetate ester, and alcohol is ethyl(S)-4-chloro-3-hydroxybutyrate.

35. The method for producing alcohol described in 30, the method further comprising converting oxidized beta-nicotinamide adenine dinucleotide to its reduced form.

36. The method for producing alcohol described in 35, wherein oxidized beta-nicotinamide adenine dinucleotide

is reduced by a conversion of glucose to delta-gluconolactone by using a glucose dehydrogenase.

37. The method for producing alcohol described in 36, wherein glucose dehydrogenase is expressed by the transformant described in 23.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern.

FIG. **2** shows the pH-dependence of the ethyl 4-chloroacetoacetate-reducing activity of the carbonyl reductase produced in Example 1. In the figure, circles represent results using Britton-Robinson buffer; squares, sodium acetate buffer; and triangles, potassium phosphate buffer.

FIG. **3** shows the temperature-dependence of the ethyl 4-chloroacetoacetate-reducing activity of the carbonyl reductase produced in Example 1.

FIG. **4** shows the pH stability of the carbonyl reductase produced in Example 1.

FIG. **5** shows the thermostability of the carbonyl reductase produced in Example 1.

FIG. **6** illustrates the restriction map of plasmid pSE-KAR1 constructed in Example 16.

FIG. **7** illustrates the restriction map of plasmid pSE420D constructed in Example 19.

FIG. **8** illustrates the restriction map of plasmid pSE-BSG1 constructed in Example 20.

FIG. **9** illustrates the restriction map of plasmid pSG-KAR1 constructed in Example 21.

DETAILED DESCRIPTION

The carbonyl reductase of this invention characteristically uses NADH as a coenzyme and reduces 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester.

In the present invention, the 4-chloroacetoacetate ester-reducing activity of the enzyme can be identified as follows.

Method of Assaying the 4-Chloroacetoacetate Ester-Reducing Activity of the Enzyme

A decrease of absorbance at 340 nm, following a decrease of NADH, is measured during the reaction at 30 in a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADH, 20 mM ethyl 4-chloroacetoacetate, and the enzyme. One unit of the enzyme of the present invention is defined as the amount required to decrease 1 mole of NADH in 1 min. A protein is quantitated by the dye-binding method using a Protein Assay Kit (BioRad).

When NADH cannot be used as a coenzyme, a decrease of absorbance at 340 nm can be only slightly observed under the conditions described above. The carbonyl reductase of the present invention shows higher activity in the presence of NADH as an electron donor instead of NADPH. Namely, NADH has significantly higher activity for the enzyme as an electron donor than NADPH. The electron donor more suitable as a coenzyme can be clearly shown by assaying the activities of the enzyme in the presence of either NADPH or NADH under the above reaction conditions and comparing the results.

The carbonyl reductase of this invention has high 4-chloroacetoacetate ester-reducing activity but does not substantially dehydrogenate (i.e., oxidize) any optical isomers of 4-halo-3-hydroxybutyrate ester. That the enzyme does not substantially dehydrogenate 4-halo-3-hydroxybutyrate ester can be judged as follows. The enzyme contacts the substrate, 4-halo-3-hydroxybutyrate ester, in the presence of NAD^+, and the change (per unit time) of the absorbance at 340 nm, which is accompanied with an increase or decrease of NADH, is measured. When the change rate is 5% or less, preferably 1% or less, taking the change of the absorbance at 340 nm where 4-chloroacetoacetate ester is used as 100%, the enzyme does not have substantial dehydrogenase activity.

In a preferred embodiment of the present invention, the carbonyl reductase does not substantially dehydrogenate isopropanol and does not reduce acetoacetate ester. That the enzyme does not have the above activities can also be judged, as described above, when the change rates of absorbance at 340 nm measured using the above substrates are 5% or less, preferably 1% or less, taking the rate where 4-chloroacetoacetate ester is used as a substrate as 100%.

The carbonyl reductase having the physicochemical properties described above is purified from, for example, cultured cells of yeast *Kluyveromyces*. In particular, *Kluyveromyces aestuarii* is preferably used in the present invention since it produces the carbonyl reductase of the present invention in a high yield. The strains of *Kluyveromyces aestuarii*, for example, *Kluyveromyces aestuarii* IFO 10597 and IFO 10598, that produce the carbonyl reductase of this invention, are available from the Institute for Fermentation, Osaka (IFO) and recited in List of Cultures 10th ed. (1996) published by IFO.

The above microorganisms are cultivated in a medium usually used for fungi such as YM medium. After sufficient cultivation, grown cells are collected and disrupted in a buffer containing a reducing agent such as 2-mercaptoethanol and a protease inhibitor such as phenylmethanesulfonyl fluoride (PMFS) to obtain cell-free extracts. The enzyme can be purified from the thus-obtained cell-free extracts by a protein solubility-dependent fractionation (precipitation by organic solvent, salting out by ammonium sulfate, etc.), and an appropriate combination of chromatography such as cation or anion exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography using chelate, dye, antibody, etc. The enzyme can be purified by, for example, hydrophobic chromatography using phenyl-Toyopearl, anion exchange chromatography using Q-sepharose, butyl-Toyopearl hydrophobic chromatography, affinity chromatography using blue sepharose, gel filtration using Superdex 200, etc. After these purification procedures, the enzyme can be electrophoretically purified to almost a single band.

In the present invention, the carbonyl reductase derived from *Kluyveromyces aestuarii* is a polypeptide having the following physicochemical properties:

(1) Reactivity

It reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced beta-nicotinamide adenine dinucleotide as an electron donor;

(2) Substrate Specificity

It has high reductase activity to 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester and

shows higher enzymatic activity when used with reduced beta-nicotinamide adenine dinucleotide as an electron donor than reduced beta-nicotinamide adenine dinucleotide phosphate.

In addition, the enzyme can have the physicochemical properties below.

(3) Optimal pH

5.0 to 6.0

(4) Substrate Specificity

The enzyme does not substantially dehydrogenate isopropanol and does not reduce acetoacetate ester.

(5) Molecular Weight

The molecular weight of the enzyme is 32,000 using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Furthermore, this enzyme can have properties described below.

(6) pH Stability

relatively stable in the range of pH 7 to 11

(7) Optimal Temperature

40 to 45

(8) Thermostability

relatively stable up to 40

(9) Inhibition

This enzyme is inhibited by SH reagents such as p-chloromercuribenzoic acid (PCMB) and copper sulfate. The enzyme is weakly inhibited by iodoacetamide but is not inhibited by quercetin or barbital.

This invention relates to an isolated nucleic acid encoding a carbonyl reductase and homologues of the enzyme. An "isolated nucleic acid" is a nucleic acid which has a non-naturally occurring sequence, or which has the sequence of part or all of a naturally occurring gene but is free of the genes that flank the naturally occurring gene of interest in the genome of the organism in which the gene of interest naturally occurs. The term therefore includes a recombinant DNA incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote. It also includes a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment. It also includes a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are mixtures of DNA molecules, vectors, or clones as they occur in a DNA library such as a cDNA or genomic DNA library. Also excluded are RNA molecules that consist of naturally-occurring sequences (e.g., naturally-occurring mRNA), except where the RNA is in a purified state such that it is at least 90% free of other naturally-occurring RNA species. Thus, a naturally-occurring mRNA in a whole mRNA preparation prepared from a cell would not be an "isolated nucleic acid," but a single mRNA species purified to 90% homogeneity from that whole mRNA preparation would be.

The nucleic acid encoding the carbonyl reductase of this invention comprises, for example, the nucleotide sequence represented by SEQ ID NO: 1. The nucleotide sequence represented by SEQ ID NO: 1 encodes a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2. Nucleic acid homologues encoding the carbonyl reductase of the present invention comprise the amino acid sequence represented by SEQ ID NO: 2 in which one or more amino acids are deleted, substituted, inserted, and/or added, and DNAs encoding the polypeptide having physicochemical properties described in (1) and (2). It will be apparent to those skilled in the art that modifications such as substitution, deletion, insertion, and/or addition may be made in the DNA described in SEQ ID NO: 1 using site-directed mutagenesis (Nucleic Acid Res. 10, pp. 6487 (1982), Methods in Enzymol. 100, pp. 448 (1983), Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press (1989), PCR A Practical Approach IRL Press pp. 200 (1991)), and the like, to obtain homologues.

The nucleic acid homologues of the present invention hybridize with a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 1 under stringent conditions, and encode a polypeptide having physicochemical properties (1) and (2) described above. DNAs that hybridize under stringent conditions are defined to be DNAs hybridizing with one or more DNA probes such as any DNA fragments sequentially containing at least 20 bases, preferably at least 30 bases, for example, 40 bases, 60 bases, or 100 bases of the DNA sequence represented by SEQ ID NO: 1 under the hybridization conditions: 37° C., 1×SSC; followed by washing at 42° C., 0.5×SSC.

The nucleic acid homologues of the present invention comprise a nucleic acid encoding a polypeptide having at least 70%, preferably at least 80% or 90%, and more preferably 95% or more homology with the amino acid sequence represented by SEQ ID NO: 2.

To determine the "percent homology (identity)" of two nucleic acids or of two amino acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first nucleic acid or amino acid sequence for optimal alignment with a second nucleic acid or amino acid sequence). The nucleotides or amino acid residues at corresponding nucleotide positions or amino acid positions are then compared. When a position in the first sequence is occupied by the same nucleotide or amino acid residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=# of identical positions/total # of positions (e.g., overlapping positions×100). In one embodiment the two sequences are the same length.

Based on the above general principles, the "percent identity" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264–2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873–5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403–410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12. BLAST searches are performed with the XBLAST program, score=50, wordlength=3. Where gaps exist between two sequences, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids Res. 25:3389–3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

The carbonyl reductase of the present invention includes a substantially pure polypeptide comprising the amino acid sequence described in SEQ ID NO: 2 and having the physicochemical properties (1) and (2) above, and its homologues. The term "substantially pure" used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological compounds, such as those in cellular material, viral material, or culture medium, with which the polypeptide may have been associated (e.g., in the course of production by recombinant DNA techniques or before purification from a natural biological source). The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example,

by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A preferable example of the polypeptide of the present invention is a polypeptide comprising the amino acid sequence described in SEQ ID NO: 2.

Homologues of the carbonyl reductase of the present invention include the amino acid sequence described in SEQ ID NO: 2 in which one or more amino acids are deleted, substituted, inserted, and/or added. It will be apparent to those skilled in the art that such modifications as substitution, deletion, insertion, and/or addition may be made in the DNA described in SEQ ID NO: 1 using site-directed mutagenesis (Nucleic Acid Res. 10, pp. 6487 (1982), Methods in Enzymol. 100, pp. 448 (1983), Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press (1989), PCR A Practical Approach IRL Press pp. 200 (1991)), etc., thereby obtaining DNAs encoding homologues of the carbonyl reductase. The DNA encoding a homologue of the carbonyl reductase is introduced into host cells and expressed to obtain the homologue of the carbonyl reductase described in SEQ ID NO: 2.

The homologues of the carbonyl reductase of the invention can also be a substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2 containing up to 30 conservative amino acid substitutions, and having the physicochemical properties (1) and (2).

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with another residue having a chemically similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

More specifically, homologues of the carbonyl reductase of the present invention are polypeptides having at least 70%, preferably at least 80% or 90%, and more preferably 95% or more homology with the amino acid sequence represented by SEQ ID NO: 2. The percent homology between the two amino acid sequences can be determined as described above. A homology search of the amino acid sequence described in SEQ ID NO: 2 was actually performed by using the BLAST program in DDBJ. As a result, Sorbitol Utilization Protein SOU1 derived from *Candida albicans* was found to have the highest homology (63%) among known proteins.

An nucleic acid encoding the carbonyl reductase of the present invention can be isolated by, for example, the method described below.

The N-terminal amino acid sequence of the purified enzyme of the present invention is determined. The enzyme is cleaved with lysyl endopeptidase, V8 protease, etc., and the peptide fragments are purified by reversed-phase liquid chromatography, etc. The amino acid sequence of the peptide fragments thus obtained can be determined by a protein sequencer.

A fragment of the nucleic acid of the present invention can be obtained in the following manner. The primers for PCR are designed based on the amino acid sequence determined. A portion of the nucleic acid of the present invention can then be amplified by PCR using the designed primers and chromosomal DNAs or cDNA libraries of cells that produce the enzyme as a template.

Furthermore, the thus-obtained nucleic acid fragments can be used to obtain the nucleic of this invention as follows. Libraries and cDNA libraries are prepared from *E. coli* transformed with a phage, plasmid, etc. into which a restriction endonuclease-digest of a chromosomal DNA from enzyme-producing strains is inserted. The thus-obtained libraries and cDNA libraries can then be used for colony hybridization and plaque hybridization using the amplified DNA as a probe, thereby obtaining the nucleic acid of this invention.

The nucleic acid of the present invention can also be obtained in the following manner. Primers for PCR are designed to extend a known DNA fragment based on the sequence obtained by analyzing the nucleotide sequences of the amplified DNA fragments. Chromosomal DNAs from the enzyme-producing strains are digested with appropriate restriction endonucleases. An inverse PCR (Genetics 120, 621–623 (1988)) is then performed using the self-circularized DNA formed from the above digests as a template and the primers. The RACE (Rapid Amplification of cDNA End, "PCR Experiment Manual" p 25–33, HBJ Academic Press) method, etc. can also be used, thereby obtaining the nucleic acid of this invention.

Thus, the nucleic acid of the present invention can be obtained by cloning genome DNA and cDNA using the above methods. Alternatively, the nucleic acid of the invention can be synthesized.

A vector expressing the enzyme of the present invention can be provided by inserting the thus-obtained nucleic acid encoding the carbonyl reductase of the invention into a known expression vector. The carbonyl reductase of the present invention can be produced by cultivating a transformant harboring this expression vector.

In the present invention, microorganisms to be transformed for expression of the carbonyl reductase requiring NADH as an electron donor are not limited as long as they can be transformed with the recombinant vector containing the nucleic acid encoding the polypeptide having the carbonyl reductase activity requiring NADH as an electron donor and can express the polypeptide. Examples of the microorganisms include those for which host-vector systems are developed, such as bacteria belonging to the genus *Escherichia, Bacillus, Pseudomonas, Serratia, Brevibacterium, Corynebacterium, Streptococcus,* or *Lactobacillus*; actinomycetes belonging to the genus *Rhodococcusor* or *Streptomyces*; yeast belonging to the genus *Saccharomyces, Kluyveromyces, Schizosaccharomyces, Zygosaccharomyces, Yarrowia, Trichosporon, Rhodosporidium, Hansenula, Pichia*, or *Candida*; fungi belonging to the genus *Neurospora, Aspergillus, Cephalosporium*, or *Trichoderma*.

The procedure for generating transformants and constructing recombinant vectors suitable for hosts can be performed according to standard techniques known in the fields of molecular biology, bioengineering, and genetic engineering (for example, Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratories). The transformants such as microorganisms stably maintaining DNA-inserted phages or plasmids should transcript and translate the genetic information so as to express the carbonyl reductase gene of the present invention requiring NADH as an electron donor. An IA promoter that regulates transcription and translation is inserted 5'-upstream of the DNA of the present invention; preferably, a terminator is also inserted 3'-downstream of the DNA. The promoter and terminator should function in microorganisms to be used as host cells. Vectors, promoters, and terminators functioning in various

microorganisms are described in "Biseibutsugaku Kisokouza (Basic Course of Microbiology) Vol. 8 Idenshikougaku (Genetic Engineering), Kyoritsu Shuppan Co., Ltd., particularly for yeast, described in "Adv. Biochem. Eng. 43, 75–102 (1990), Yeast 8, 423–488 (1992)" etc.

For example, plasmid vectors such as pBR and pUC series, and promoters such as those of -galactosidase (lac), tryptophan operon (trp), tac, trc (fusion of lac and trp), and those derived from -phage PL, PR, etc. can be used for the genus Escherichia, particularly *Escherichia coli*. Terminators derived from trpA, phage, and rrn ribosomal RNA can also be used.

Vectors such as the pUB110 and pC194 series can be used for the genus *Bacillus* and can be integrated into chromosomes. Promoters and terminators such as those of alkaline protease (apr), neutral protease (npr), and -amylase (amy) can be used.

Host-vector systems for the genus *Pseudomonas*, specifically *Pseudomonas putida* and *Pseudomonas cepacia*, have been developed. A broad host range vector pKT240 (containing genes necessary for autonomous replication derived from RSF1010) based on plasmid TOL that is involved in degradation of toluene compounds can be utilized. A promoter and terminator of a lipase (JP-A Hei 5-284973) gene and the like can be used.

Plasmid vectors such as pAJ43 (Gene 39, 281 (1985)) can be used for the genus *Brevibacterium*, especially *Brevibacterium lactofermentum*. Promoters and terminators for the genus *Escherichia* can be used for this microorganism.

Plasmid vectors such as pCS11 (JP-A Sho 57-183799) and pCB101 (Mol. Gen. Genet. 196, 175 (1984)) can be used for the genus *Corynebacterium*, particularly, *Corynebacterium glutamicum*.

Plasmid vectors such as pHV1301 (FEMS Microbiol. Lett., 26, 239 (1985)) and pGK1 (Appl. Environ. Microbiol. 50, 94 (1985)) can be used for the genus *Streptococcus*.

For the genus *Lactobacillus*, pAM 1 developed for the genus *Streptococcus* (J. Bacteriol. 137, 614 (1979)) can be used, and some of the promoters for the genus *Escherichia* are applicable.

For the genus *Rhodococcus*, a plasmid vector isolated from *Rhodococcus rhodochrous* can be used (J. Gen. Microbiol. 138, 1003 (1992)).

Plasmids functioning in the genus *Streptomyces* can be constructed by the method described in "Genetic Manipulation of Streptomyces: A Laboratory Manual Cold Spring Harbor Laboratories by Hopwood et al. (1985)." For example, pIJ486 (Mol. Gen. Genet. 203, 468–478 (1986)), pKC1064 (Gene 103, 97–99 (1991)), and pUWL-KS (Gene 165, 149–150 (1995)) can be used, particularly for *Streptomyces lividans*. Such plasmids can also be used for *Streptomyces virginiae* (Actinomycetol. 11, 46–53 (1997)).

Plasmids such as the YRp, YEp, YCp, and YIp series can be used for the genus *Saccharomyces*, especially for *Saccharomyces cerevisiae*. Integration vectors (such as EP 537456) using homologous recombination with multiple copies of a ribosomal DNA in genomic DNA are extremely useful because they are capable of introducing multiple copies of genes into the host genome and stably maintaining the genes. Furthermore, promoters and terminators of alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acid phosphatase (PHO), -galactosidase (GAL), phosphoglycerate kinase (PGK), enolase (ENO), etc. can be used.

Plasmids such as the series of 2 m plasmids derived from *Saccharomyces cerevisiae*, the series of pKD1 plasmids (J. Bacteriol. 145, 382–390 (1981)), plasmids derived from pGK11 involved in killer activity, the series of KARS plasmids containing an autonomous replication gene from the genus *Kluyveromyces*, and vector plasmids (such as EP 537456) capable of being integrated into chromosomes by homologous recombination with ribosomal DNA can be used for the genus *Kluyveromyces*, particularly for *Kluyveromyces lactis*. Promoters and terminators derived from ADH and PGK are applicable.

For the genus *Schizosaccharomyces*, plasmid vectors containing ARS (a gene involved in autonomous replication) derived from *Schizosaccharomyces pombe* and containing selective markers supplementing auxotrophy of *Saccharomyces cerevisiae* can be used (Mol. Cell. Biol. 6, 80 (1986)). Furthermore, ADH promoter derived from *Schizosaccharomyces pombe* is applicable (EMBO J. 6, 729 (1987)). In particular, pAUR224 is commercially available from Takara Shuzo.

For the genus *Zygosaccharomyces*, plasmid vectors such as pSB3 (Nucleic Acids Res. 13, 4267 (1985)) derived from *Zygosaccharomyces rouxii* can be used. Promoters of PHO5 derived from *Saccharomyces cerevisiae* and glycerolaldehyde-3-phosphate dehydrogenase (GAP-Zr) derived from *Zygosaccharomyces rouxii* (Agri. Biol. Chem. 54, 2521 (1990)), etc. are available.

For the genus *Hansenula*, host-vector systems have been developed for *Hansenula polymorpha*. Genes (HARS1, HARS2) involved in autonomous replication derived from *Hansenula polymorpha* can be used as vectors. These genes cannot be maintained stably, so multiple copies of them should be integrated into chromosomes (Yeast 7, 431–443 (1991)). Promoters of alcohol oxidase (AOX) that is induced by methanol and the like and formic acid dehydrogenase (FDH) can be used.

For the genus *Pichia*, host-vector systems for *Pichia pastoris* have been developed using genes such as PARS1 and PARS2 involved in autonomous replication derived from *Pichia* (Mol. Cell. Biol. 5, 3376 (1985)). Promoters such as a promoter of AOX with strong promoter activity induced by high-density culture and methanol are applicable (Nucleic Acids Res. 15, 3859 (1987)).

For the genus *Candida*, host-vector systems have been developed for *Candida maltosa, Candida albicans, Candida tropicalis, Candida utilis*, etc. Vectors for *Candida maltosa* using ARS, which was cloned from this strain, have been developed (Agri. Biol. Chem. 51, 51, 1587 (1987). Strong promoters for vectors that are able to be integrated into chromosomes have been developed for *Candida utilis* (JP-A Hei 08-173170).

In the genus *Aspergillus, Aspergillus niger* and *Aspergillus oryzae* have been most extensively studied. Plasmids able to be integrated into chromosomes are available. Promoters derived from extracellular protease and amylase are available (Trends in Biotechnology 7, 283–287 (1989)).

For the genus *Trichoderma*, host-vector systems based on *Trichoderma reesei* have been developed, and promoters derived from extracellular cellulase genes are available (Biotechnology 7, 596–603 (1989)).

Various host-vector systems for plants and animals, in addition to microorganisms, have been developed. In particular, expression systems for producing a large amount of foreign proteins in insects, particularly silkworms (Nature 315, 592–594 (1985)), and plants such as rape seeds, corns, and potatoes have been developed and are available.

In the present invention, microorganisms capable of producing the enzyme that reduces 4-haloacetoacetate ester include all strains belonging to the genus *Kluyveromyces*, their mutants and variants capable of producing the NADH-

dependent carbonyl reductase, and genetically engineered transformants that have acquired the capacity of producing the enzyme.

The present invention relates to the production of alcohol by reducing ketone using the above carbonyl reductase. The carbonyl reductase of this invention is industrially advantageous since it can use NADH as a coenzyme, which is less expensive and more stable than NADPH. The desired enzymatic reaction can be performed by contacting the reaction solution with the enzyme molecules, their treated products, cultured broth containing the enzyme molecules, or living transformants such as microorganisms producing the enzymes. Furthermore, the contact of the reaction solution with the enzyme is not limited thereto. The reaction solution is defined as an appropriate solvent containing a substrate and NADH that is a coenzyme necessary for the enzymatic reaction, wherein the solvent provides circumstances desirable for the enzyme to express the enzyme activity. The treated microorganisms containing the carbonyl reductase of the present invention include microorganisms whose cell membrane permeability is modified by treating them with surfactants or organic solvents such as toluene, cell-free extracts obtained by disrupting the microorganisms with glass beads, or by treating them with enzyme, the partially purified preparation of the extracts, etc. The microbial cells can also be used by being immobilized on carageenan gel, alginate gel, polyacrylamide gel, cellulose, agar, or the like supporting material, using a known method.

The ketone used in the method for producing alcohol according to the present invention, 2,3-butandione, with neighboring diketones and 4-haloacetoacetate ester derivatives is preferable. Halogen atoms of ethyl 4-haloacetoacetate derivatives include bromine, iodine, and chlorine, but preferably chlorine. Examples of esters include those of alcohol having linear chains, branched chains, and aromatic substitutions, such as methyl, ethyl, propyl, isopropyl, butyl, octyl, and benzyl esters. Ethyl ester is most preferable. The 4-haloacetoacetate ester derivatives include, for example, those with alkyl groups containing a linear chain or branched chain at the 2-position and halogens such as chlorine, bromine, and iodine.

NAD^+ formed from NADH during reductive reactions can be converted into NADH using the NAD^+-reduction ability of microorganisms (glycolytic pathway, catabolic pathway of C1 compounds in methylotroph). The ability of the NAD^+-reduction can be enhanced by adding glucose, ethanol, formic acid, etc. to a reaction system. Furthermore, the above enhancement can be achieved by adding microorganisms, their treated products, or their enzymes capable of forming NADH from NAD^+ to the reaction system. For example, microorganisms having glucose dehydrogenase, formate dehydrogenase, alcohol dehydrogenase, amino acid dehydrogenase, or organic acid dehydrogenase (malate dehydrogenase and so on); their treated products; their enzymes that are partially or highly purified can be used. The reactants necessary for regenerating NADH can be added to the reaction system for producing alcohol of the present invention as they are, in immobilized forms, or through a membrane capable of regenerating NADH.

Such additional reaction systems for regenerating NADH are unnecessary when living transformants containing a recombinant vector harboring the DNA of this invention are used in the above-described method for producing alcohol. Namely, NADH is efficiently regenerated without adding the enzyme for NADH regeneration in the reduction reaction with transformant microorganisms if the microorganisms have high NADH regenerating activity. It is also possible to express the NADH-regenerating enzyme and NADH-dependent carbonyl reductase and to perform the reduction reaction using them more efficiently by introducing a gene encoding the NADH-regenerating enzyme such as glucose dehydrogenase, formate dehydrogenase, alcohol dehydrogenase, amino acid dehydrogenase, or organic acid dehydrogenase (malate dehydrogenase and so on) to a host cell together with the DNA encoding the NADH-dependent carbonyl reductase of this invention. These two or more genes can be introduced into a host by transforming a host with recombinant vectors each having different replication origin and each enzyme gene to avoid incompatibility, transforming a host with a single vector into which the genes are inserted, or introducing the genes into host chromosomes. When two or more genes are introduced into a single vector, each gene is ligated with gene expression-regulating regions including promoters and terminators. Alternatively, the genes are expressed as an operon containing two or more cistrons such as a lactose operon.

The reduction reaction using the enzyme of the present invention can be performed in water; a water-insoluble organic solvent such as ethyl acetate, butyl acetate, toluene, chloroform, or n-hexane; or a two-phase system of such a solvent and an aqueous solvent.

The reaction of this invention can be performed using an immobilized enzyme, a membrane reactor, etc.

The reaction of this invention can be performed at temperatures ranging from 4 to 60, preferably 10 to 37; a pH ranging from 3 to 11; preferably 5 to 8; and the concentration of substrates ranging from 0.01 to 90 w/v %, preferably 0.1 to 30 w/v % based on the reaction mixture. NAD^+ or NADH can be added to the reaction system as a coenzyme in a concentration ranging from 0.001 to 100 mM, preferably 0.01 to 10 mM. The substrate can be added once at the beginning of the reaction. Preferably, it is added continuously or several times with divided portions to prevent the concentration of the substrate from becoming too high in the reaction system.

Compounds added to the reaction system for regenerating NADH, for example, glucose in case of using glucose dehydrogenase, formic acid in case of using formate dehydrogenase, and ethyl alcohol or isopropanol in case of using alcohol dehydrogenase, can be added at a molar ratio of 0.1 to 20, and preferably 1 to 5 or more per mole of the substrate, ketone. The enzymes for regenerating NADH, for example, glucose dehydrogenase, formate dehydrogenase, and alcohol dehydrogenase, can be added in an amount of 0.1- to 100-fold, and preferably 0.5- to 20-fold, enzymatic activity based on that of the NADH-dependent carbonyl reductase of this invention.

Alcohol formed by the reduction reaction of ketone according to the present invention can be purified from the cells and proteins by appropriate combinations of known purification techniques such as centrifugation, membrane treatment, solvent extraction, or distillation.

For example, the cells are removed from the reaction solution by centrifugation, proteins are removed by ultrafiltration, and solvents such as ethyl acetate and toluene are added to the filtrate to extract 4-halo-3-hydroxyacetate ester into the solvent layer. The organic solvent layer is subjected to phase separation and distilled to obtain 4-halo-3-hydroxyacetate ester with high purity.

The NADH-dependent carbonyl reductase and the DNA encoding the enzyme of the present invention are advantageous in the industrial production of alcohols. The method of the present invention using the above enzyme enables

efficiently producing (S)-4-halo-3-hydoxybutyrate ester with high optical purity.

The following examples further illustrate this invention but are not to be construed to limit the scope of the invention.

EXAMPLE 1

Purification of Carbonyl Reductase

Kluyveromyces aestuarii IFO 10597 was cultured in 20 L of YM medium containing 24 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, and 5 g/L peptone (pH 6.0). The cultured cells were harvested by centrifugation. The wet cells thus obtained were suspended in 50 mM potassium phosphate buffer (pH 8.0) containing 0.02% 2-mercaptoethanol and 2 mM phenylmethanesulfonyl fluoride (PMSF). The suspended cells were disrupted with a bead beater (BioSpec Co.), then cell debris was removed by centrifugation to obtain cell-free extracts. After protamine sulfate was added to the thus-obtained cell-free extract, the mixture was centrifuged to obtain a nucleic acid-free supernatant. The supernatant was adjusted to 25% saturation with ammonium sulfate and was applied to a phenyl-Toyopearl 650M column (5.0 cm×27 cm) equilibrated with a standard buffer (10 mM potassium phosphate buffer (pH 8.0), 0.01% 2-mercaptoehanol) containing 25% ammonium sulfate. Elution was then performed with a linear gradient of ammonium sulfate solution from a concentration of 25 to 0%.

The activity of the carbonyl reductase was detected in three fractions, a passed-through fraction and two gradient-eluted fractions. The latest fraction among the three fractions was pooled and concentrated by ultrafiltration.

After the concentrated enzyme solution was dialyzed against a standard buffer, the dialysate was applied to a Q-sepharose HP column (2.6 cm×10 cm) equilibrated with the standard buffer. The column was subsequently washed with the standard buffer and the standard buffer containing 0.2 M NaCl. Elution was performed with a linear gradient of NaCl solution from 0.2 to 0.9 M. Active eluted fractions were pooled and concentrated by ultrafiltration.

The concentrated enzyme solution was adjusted to 20% saturation with ammonium sulfate and was applied to a butyl-Toyopearl 650M column (1.6 cm×5 cm) equilibrated with the standard buffer containing 20% saturation ammonium sulfate. The column was washed with the same buffer, and elution was performed with a linear gradient of ammonium sulfate solution from a concentration of 20 to 0%. The peak of the activity of the carbonyl reductase was detected in two eluted fractions. The first fraction with major activity was pooled.

After the concentrated active fraction was dialyzed against the standard buffer, the enzyme solution was loaded on a Blue-sepharose HP column (1.6 cm×2.5 cm) equilibrated with the same buffer, and the column was then washed with the standard buffer. Elution was performed with a linear gradient of NaCl solution from 0 to 1 M. The passed-through fraction in which the activity of the carbonyl reductase was detected was pooled and concentrated. The concentrated enzyme solution was subjected to gel filtration with Superdex 200 column (1.6 cm×100 cm using the standard buffer containing 0.3 M NaCl).

The active fractions obtained by gel filtration were concentrated and analyzed by polyacrylamide gel electrophoresis (PAGE). The enzyme was detected as a substantially single band (FIG. **1**).

The specific activity of the purified enzyme was about 28.6 U/mg. The purification steps of the enzyme are shown in Table 1.

TABLE 1

Purification step	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg)
Cell-free extract	55700	3610	0.0648
Protamin sulfate precipitation	28500	4730	0.166
Phenyl-Toyopearl	591	83.2	0.141
Q-Sepharose	50.0	42.8	0.856
Butyl-Toyopearl	3.62	23.6	6.52
Blue-sepharose	0.21	3.00	14.3
Superdex 200	0.021	0.60	28.6

EXAMPLE 2

Molecular Weight of Carbonyl Reductase

The molecular weight of the subunit of the enzyme obtained in Example 1 was determined to be 32 kDa by SDS-PAGE. The molecular weight determined by gel filtration using Superdex G200 was approximately 85 kDa.

EXAMPLE 3

Optimal pH of the Carbonyl Reductase

The relative reductase activity of the enzyme obtained in Example 1 to ethyl 4-chloroacetoacetate was investigated with varying pH values of the reaction solution using the potassium phosphate buffer, the sodium acetate buffer, and the Britton-Robinson buffer. The results are shown in FIG. **2** as activity relative to the maximum activity that is regarded as 100. The optimal pH of the reaction was estimated to be pH 5.0 to 6.0.

EXAMPLE 4

Optimal Temperature of the Carbonyl Reductase

The reductase activity of the enzyme obtained in Example 1 with respect to ethyl 4-chloroacetoacetate was determined under the standard reaction condition with varying reaction temperatures. The results are shown in FIG. **3** as the activity relative to the maximum activity that is regarded as 100. The optimal temperature was determined to be 40 to 45.

EXAMPLE 5

pH Stability of the Carbonyl Reductase

The enzyme obtained in Example 1 was incubated at 30 for 30 min in the Briiton-Robinson buffer, ranging from pH 4 to 12, and the activities were measured. The results are shown in FIG. **4** as the residual activity to the activity of the untreated enzyme that is regarded as 100. The most stable pH of the carbonyl reductase of the present invention was determined to be pH 7.0 to 11.0.

EXAMPLE 6

Thermostability of the Carbonyl Reductase

The enzyme obtained in Example 1 was incubated at pH 8.0 for 10 min at 30, 37, 45, 50, and 55; the reductase activity of each incubation mixture to ethyl 4-chloroacetoacetate was then measured. The results are shown in FIG. **5** as the residual activity to the activity of the untreated enzyme that is taken as 100. The carbonyl reduc-

tase of the present invention showed 75% or more of the residual activity up to 37.

EXAMPLE 7

Substrate Specificity of the Carbonyl Reductase

The reductase activity of the enzyme obtained in Example 1 was measured by reacting the enzyme with various ketones and aldehydes in the presence of a coenzyme. The results are shown in Table 2 as the activity relative to that of ethyl 4-chloroacetoacetate in the presence of NADH that is taken as 100. The carbonyl reductase of the present invention showed only 6.4% of the activity in the presence of NADPH as a coenzyme to that in the presence of NADH that is taken as 100%. The enzyme showed that the relative activity to 2,3-butadione used as ketone was 70.6%. This enzyme did not show any NAD^+-dependent dehydroganase activity to ethyl(R) or (S)-4-chloro-3-hydroxybutyrate.

TABLE 2

Substrate	Coenzyme	Relative activity (%)
Reduction reaction		
ethyl 4-chloroacetoacetate	NADH	100
	NADPH	6.4
methyl 4-chloroacetoacetate	NADH	13.1
ethyl acetoacetate	NADH	0
methyl acetoacetate	NADH	0
2,3-butandione	NADH	70.6
2,3-pentandione	NADH	8.2
2,4-pentandione	NADH	0
Propionaldehyde	NADH	2.5
pyridin-3-aldehyde	NADH	0
Methylglyoxal	NADH	0
Acetophenone	NADH	0
Oxidation reaction		
ethyl (R)-4-chloro-3-hydroxybutyrate	NAD^+	0
ethyl (S)-4-chloro-3-hydroxybutyrate	NAD^+	0
ethyl (R)-3-hydroxybutyrate	NAD^+	1.9
ethyl (S)-3-hydroxybutyrate	NAD^+	0
Isopropanol	NAD^+	0

EXAMPLE 8

Influence of the Carbonyl Reductase Inhibitors

The enzyme obtained in Example 1 was incubated at 30 for 10 min in the presence of various reagents, and its reductase activity to ethyl 4-chloro-3-hydroxybutyrate was measured. The results are shown in Table 3 as the residual activity to that obtained without any reagent that is taken as 100. The carbonyl reductase of this invention was significantly inhibited by p-chloromercuribenzoic acid and copper sulfate, but not by quercetin and barbital.

TABLE 3

Inhibitor	Concentration	Residual activity
		100
p-chloromercuribenzoic acid	0.05 mM	0
iodoacetamide	1 mM	84.0
copper sulfate	1 mM	19.0
quercetin	0.1 mM	94.0
barbital	1 mM	101
Phenylmethanesulfonyl fluoride	1 mM	99.0

EXAMPLE 9

Synthesis of ethyl(S)-4-chloro-3-hydroxybutyrate by the Carbonyl Reductase [1]

The reaction was carried out at 30 overnight in a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 139.8 mg of NADH, 0.5 U of the carbonyl reductase, and 2% ethyl 4-chloro-3-hydoroxyacetoacetate. The optical purity of the formed ethyl 4-chloro-3-hydoroxybutyrate was measured as follows. Ethyl 4-chloro-3-hydoroxybutyrate was extracted from the reaction mixture with ethyl acetate. After the solvent was removed from the extract, the extract was subjected to a liquid chromatography using an optical resolution column, Chiralcel OD column (4.6 mm×25 cm, Daicel Chemical Industries, Co., Ltd.), using a mixture of n-hexane and isopropanol (9:2) as an eluent at a flow rate of 0.5 mL/min, monitored with RI. As a result, ethyl 4-chloro-3-hydoroxybutyrate produced by the method of this invention was S-isomer with an optical purity of 99% ee or more.

The yield of ethyl 4-chloro-3-hydoroxybutyrate formed from ethyl 4-chloro-3-hydroxyacetoacetate as the starting material was measured by gas chromatography. The gas chromatography was performed at 150 using a Thermon-3000 5% Chromosorb W 60–80 (AW-DMCS)(3.2 mm×210 cm, Shinwa Chemical Industries, Ltd.) and a hydrogen flame ionization detector (FID). The reaction yield was about 95%.

EXAMPLE 10

Synthesis of ethyl(S)-4-chloro-3-hydroxybutyrate by the Carbonyl Reductase [2]

The reaction was carried out at 30 overnight in 1 ml of the reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 1 mM NAD^+, 0.5 U of the carbonyl reductase obtained in Example 1, 2% ethyl 4-chloroacetoacetate, 1 U of glucose dehydrogenase (Wako Pure Chemical Industries, Ltd.), and 250 mM glucose. The formed ethyl 4-chloro-3-hydoroxybutyrate was S-isomer with an optical purity of 99% ee or more. The yield of the reaction was about 95% (analyzed by the same method as in Example 9).

EXAMPLE 11

Analysis of Partial Amino Acid Sequence of the Carbonyl Reductase

The carbonyl reductase obtained in Example 1 was subjected to SDS-PAGE. A piece of gel containing the enzyme was cut out, washed twice with 0.2M $(NH_3)_2CO_3$, and then subjected to in-gel-digestion at 35 overnight using lysyl endopeptidase. The digested peptides were subjected to a reversed-phase HPLC (TSK gel ODS-80-Ts, 2.0 mm×250 mm, Tosoh Corporation), eluted with a gradient of acetonitrile solution in 0.1% trifluoroacetic acid (TFA), and fractionated.

Three kinds of the thus-obtained peptide fractions were named lep50_51, lep54, and lep59, and an amino acid sequence of each protein was determined by a protein sequencer (Hewlett Packard G1005A Protein Sequencer System). Amino acid sequences of Lep50_51, lep54, and lep59 are described in SEQ ID Nos: 3, 4, or 5, respectively.

EXAMPLE 12

Purification of the Chromosomal DNA from *Kluyveromyces aestuarii*

The *Kluyveromyces aestuarii* IFO 10597 strain was cultured in YM medium, and the cells were separated. The chromosomal DNA was purified from the cells according to the method described in Meth. Cell Biol. 29, 39–44 (1975).

EXAMPLE 13

Cloning of the Carbonyl Reductase Gene by PCR

Six kinds of sense primers and anti-sense primers were synthesized based on amino acid sequences lep50_51,

lep54, and lep59. Nucleotide sequences are described in SEQ ID NO: 6 (KAR50-S), SEQ ID NO: 7 (KAR50-A), SEQ ID NO: 8 (KAR54-S), SEQ ID NO: 9 (KAR54-A), SEQ ID NO: 10 (KAR59-S), and SEQ ID NO: 11 (KAR59-A).

One sense primer and one anti-sense primer were paired to select a total of six primer pairs, and PCR was performed for 30 cycles in a reaction consisting of denaturation (94, 30 sec), annealing (45, 30 sec), and extension (70, 1 min), using GeneAmp PCR System 2400 (Perkin Elmer) in 50 L of the reaction mixture containing 50 pmol each primer, 10 nmol dNTP, 50 ng of the chromosomal DNA from *Kluyveromyces aestuarii*, the buffer for AmpliTaq (Takara Shuzo), and 2U AmpliTaq (Takara Shuzo).

A portion of the PCR reaction mixture was analyzed by agarose gel electrophoresis. As a result, putative specific bands were detected for the combinations of KAR50-S and KAR54-A, KAR50-S and KAR59-A, and KAR54-S and KAR59-A.

EXAMPLE 14

Subcloning of PCR Fragments of the Carbonyl Reductase Gene

The three DNA fragments obtained in Example 13 were extracted with phenol/chloroform and precipitated with ethanol to recover them as precipitates. Each of the DNA fragments was digested with BamHI and HindIII restriction endonucleases and subjected to agarose gel electrophoresis. A piece of agarose gel containing the target band was cut out, purified by SUPREC-01 (Takara Shuzo), and recovered as ethanol precipitates.

The DNA fragments thus obtained were ligated with plasmid pUC18, which was digested with BamHI and HindIII, using a Takara Ligation Kit, and *Escherichia coli* JM109 was then transformed with the ligated plasmid.

The transformants were grown on the LB plate (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) supplemented with ampicillin (50 g/mL), and the sizes of the inserted DNAs were confirmed by performing Colony Direct PCR for some grown colonies using M13-21 and M13-RP primers. The putative colonies harboring the target DNA fragments were cultured in LB liquid medium containing ampicillin, and the plasmids were then purified from the grown cells with Flexi-Prep (Pharmacia). The plasmids obtained by PCR using a combination of KAR50-S and KAR54-A, that of KAR50-S and KAR59-A, and that of KAR54-S and KAR59-A were named pKAR1, pKAR2, and pKAR3, respectively.

The purified plasmids were digested with BamHI and HindIII restriction endonucleases to confirm the sizes of the target inserts, followed by sequencing. Namely, the inserted DNAs were subjected to PCR using Dye Terminator Cycle Sequencing FS ready Reaction Kit (Perkin Elmer), and sequenced by DNA Sequencer 373A (Perkin Elmer). Nucleotide sequences of inserted fragments of pKAR1, pKAR2, and pKAR3 are described in SEQ ID NOs: 12, 13, and 14, respectively.

EXAMPLE 15

Subcloning DNA Near the Carbonyl Reductase Gene

The chromosomal DNA from *Kluyveromyces aestuarii* was digested with HaeII, and the digests were self-ligated at 16 overnight using T4 DNA ligase to circularize each DNA fragment. The circularized DNAs were subjected to PCR for 30 cycles of a reaction consisting of denaturation (94, 30 sec), annealing (55, 30 sec), and extension (70, 5 min) in 50 L of the reaction mixture containing 50 pmol each of primers KAR-5UP and KAR-3DN, 10 nmol of dNTP, 50 ng of the circularized DNAs, a buffer for Ex-Taq (Takara Shuzo), and 3U of Ex-Taq (Takara Shuzo) with GeneAmp PCR System 2400 (Perkin Elmer). A 9.5 kb fragment was detected by analyzing a portion of the PCR products by agarose gel electrophoresis.

The nucleotide sequences of primers KAR-5UP and KAR-3DNA are shown as SEQ ID NOs: 15 and 16, respectively. The amplified DNA fragments were recovered as ethanol precipitates after extraction with phenol/chloroform. After agarose gel electrophoresis, a piece of gel containing the target band was cut out and purified using EASYTRAP Ver.2 (Takara Shuzo).

The thus-obtained DNA fragments were ligated with pT7Blue T-vector (Novagen) using Takara Ligation Kit ver. 2, and *E. coli* JM109 was transformed with the ligated plasmids. The transformants were grown on the LB plate (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) supplemented with 50 g/mL of ampicillin, 50 g/mL of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside, and 20 g/mL of isopropylthio-D-galactopyranoside (IPTG). The sizes of the inserted fragments were confirmed by performing Colony Direct PCR for several white colonies using KAR-3DN and KAR-5UP as primers. The putative colonies harboring the target DNA fragments were cultured in LB liquid medium containing ampicillin; the plasmids were then purified from the grown cells with Flexi-Prep (Pharmacia). The plasmids thus obtained were named pT7B-F3 and pT7B-F8.

The nucleotide sequences of the inserted DNAs were analyzed by the primer-walking method. The nucleotide sequence was determined by PCR using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer) and by DNA Sequencer 373A (Perkin Elmer).

The nucleotide sequence inserted into pKAR2 with its 5'-upstream and 3'-downstream sequences was synthesized and shown in SEQ ID NO: 17. The sequence of the carbonyl reductase (KaCR1) was determined by ORF search in the nucleotide sequence represented by SEQ ID NO: 17. The determined nucleotide sequence and the deduced amino acid sequence are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The above synthesis of the nucleotide sequence and OFR search were performed using Genetyx ATSQ, and Genetyx (Software Development Co., Ltd.).

EXAMPLE 16

Cloning the Carbonyl Reductase Gene

KAR-ORF5 (SEQ ID NO: 18) and KAR-ORF3 (SEQ ID NO: 19) primers for cloning were synthesized based on the nucleotide sequence of the structural gene of the carbonyl reductase. The chromosomal DNA from *Kluyveromyces aestuarii* was subjected to PCR for 30 cycles of a reaction consisting of denaturation (95", 30 sec), annealing (50", 1 min), and extension (75", 5 min) in 50" 1 of the reaction mixture containing 50 pmol of each primer, 10 nmol of dNTP, 50 ng of the chromosomal DNA from *Kluyveromyces aestuarii*, 2U of Pfu-DNA polymerase, and a buffer for the enzyme (STRATAGENE) using GeneAmp PCR System 2400 (Perkin Elmer).

The putative specific bands were observed by analyzing a portion of PCR products by agarose gel electrophoresis.

The amplified DNA fragments were recovered as ethanol precipitates after extraction with phenol/chloroform. The DNA fragments were digested with BamHI and XbaI and were subjected to agarose gel electrophoresis. A piece of agarose gel containing targeted bands was then cut out, and the DNAs were recovered as ethanol precipitates after purification using SUPREC-01 (Takara Shuzo).

The thus-obtained DNA fragments were ligated into plasmid pSE420 (Invitrogen), which was digested with BamHI and XbaI, using a Takara Ligation Kit, and the resulting plasmids were introduced into *Escherichia coli* JM109.

The transformants were grown on the LB plate containing ampicillin (50 "g/mL), and the sizes of the inserted fragments were confirmed by performing Colony Direct PCR for several grown colonies using Trc-3b and Trc-5b (SEQ ID NOs: 20 and 21) as primers. The putative colonies harboring the target DNA fragment were incubated at 30" overnight in LB liquid medium containing ampicillin; cultivation was then continued for four more hours after the addition of 0.1 mM IPTG to the culture medium.

The cells were harvested by centrifugation then suspended in 50 mM potassium phosphate buffer (pH 6.5) containing 0.02% 2-mercaptoethanol, 2 mM PMSF, and 0.5 M NaCl and disrupted by sonication for 3 min using a sealed ultrasonic cell disrupter (UCD-200™ (Cosmo Bio)). The resulting suspension was then centrifuged to obtain the supernatant as cell extracts. The cell extracts were reacted with ethyl 4-chloroacetoacetate to measure the reductase activity to the substrate.

The transformants capable of expressing the highest activity were cultured in the liquid LB medium containing ampicillin (50 "g/mL). The plasmid was purified from the transformants using Qiagen 500 (Qiagen) and named plasmid pSE-KAR1. The nucleotide sequence of the inserted DNA in the plasmid was determined by PCR using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer) and DNA Sequencer 373A (Perkin Elmer).

The inserted DNA fragment was the same sequence as that of KaCR1 represented by SEQ ID NO: 1 with 5'-upstream 12 base pairs that were attached for cloning. The nucleotide sequence of the inserted DNA fragment in plasmid pSE-KAR1, the amino acid sequence of the coding sequence, and a restriction map of the plasmid are shown in SEQ ID NO: 22, SEQ ID NO: 23, and FIG. **6**, respectively. *E. coli* transformed with the control plasmid pSE420 (not harboring the insert) was cultured overnight in LB medium. After 0.1 mM IPTG was added, cultivation was continued for an additional 4 hours, and the cells were disrupted in the same manner as described above. The reductase activity of the cell extract to ethyl 4-chloroacetoacetate was assayed, but not detected.

EXAMPLE 17

Synthesis of ethyl(S)-4-chloro-3-hydroxybutyrate by the Recombinant Carbonyl Reductase

Ethyl(S)-4-chloro-3-hydroxybutyrate was produced from ethyl 4-chloroacetoacetate using the cell extract prepared in Example 16. The reaction was performed at 25" overnight in 1 ml of the reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 1 mM NAD^+, 1.0U of the recombinant carbonyl reductase, 2% ethyl 4-chloroacetoacetate, 2 U of glucose dehydrogenase (Wako Pure Chemical Industries), and 250 mM glucose. The formed ethyl(S)-4-chloro-3-hydroxybutyrate was S-isomer with an optical purity of 98.4% ee or more, and the yield was about 99% (measured by the same methods as in Example 9).

EXAMPLE 18

Purification of the DNA from *Bacillus subtilis*

Bacillus subtilis BGSC 1A1 strain was cultured on LB medium, and the cells were harvested. The chromosomal DNA was purified from the cells using Qiagen Genomic Tip (Qiagen) according to the method described in the appendix.

EXAMPLE 19

Construction of Plasmid pSE420D for Coexpression

Plasmid vector pSE420 (Invitrogen) was digested with NcoI and BamHI, treated with Klenow fragment, and self-circularized to construct plasmid pSE420B. Synthetic DNAs SE420D-S described in SEQ ID NO: 24 and SE420D-A described in SEQ ID NO: 25 were annealed and ligated into plasmid pSE420B, which was digested using MunI and SpeI, with T4 DNA ligase, thereby obtaining plasmid pSE420D. A restriction map of plasmid pSE420D is shown in FIG. **7**.

EXAMPLE 20

Cloning of Glucose Dehydrogenase Gene from *Bacillus subtilis*

A glucose dehydrogenase gene, which is to be used for regenerating NADPH, was cloned from *Bacillus subtilis* by a known method (J. Bacteriol. 166, 238–243 (1986)).

Primers BSG-ATG1 (SEQ ID NO: 26) and BSG-TAA2 (SEQ ID NO: 27) were synthesized based on 5'- and 3'-sequences of the structural gene of the sequence of the glucose dehydrogenase gene described in the above reference, to clone only the open reading frame by PCR. PCR was performed for 30 cycles (95", 30 sec; 50", 1 min; 75", 3 min 15 sec), using the chromosomal DNA from *Bacillus subtilis* prepared in Example 18 as a template to obtain amplified DNAs.

The amplified DNA fragments were digested with EcoRI and HindIII, and ligated into a plasmid vector, pSE420D, which was digested using EcoRI and HindIII, with T4 DNA ligase to obtain plasmid pSE-BSG1. A restriction map of the plasmid is illustrated in FIG. **8**. The determined nucleotide sequence of the inserted DNA fragment agreed with the nucleotide sequence registered in the database (DDBJ Accession No. M12276). The thus-obtained nucleotide sequence of the glucose dehydrogenase gene and amino acid sequence of the protein encoded by the gene are shown in SEQ ID NO: 28 and SEQ ID NO: 29.

EXAMPLE 21

Construction of Plasmid pSG-KAR1 Coexpressing the Carbonyl Reductase from *Kluyveromyces aestuarii* and Glucose Dehydrogenase from *Bacillus subtilis*

The DNA fragments containing a gene encoding the carbonyl reductase from *Kluyveromyces aestuarii* were prepared by digesting plasmid pSE-KAR1 constructed in Example 16 with BamHI and XbaI.

The plasmid pSE-KAR1 containing the carbonyl reductase gene from *Kluyveromyces aestuarii* was digested with

BamHI and XbaI. It was then ligated with plasmid pSE-BSG1 from Example 20 containing the *Bacillus subtilis*-derived glucose dehydrogenase gene, which was digested with BamHI and XbaI, with T4 DNA ligase to obtain the plasmid pSG-KAR1. This plasmid (pSG-KAR1) can express glucose dehydrogenase and "-ketoacyl-ACP reductase simultaneously.

A restriction map of plasmid pSG-KAR1 is illustrated in FIG. **9**.

EXAMPLE 22

Coexpression of the Glucose Dehydrogenase Derived from *Bacillus subtilis* and the Carbonyl Reductase Derived from *Kluyveromyces aestuarii* in *E. coli*

E. coli JM109, HB101, TG1, and W3110 were transformed with plasmid pSG-KAR1 that can coexpress the glucose dehydrogenase from *Bacillus subtilis* and the carbonyl reductase from *Kluyveromyces aestuarii*.

Each recombinant *E. coli* strain was cultured at 30" overnight in the LB liquid medium. After 0.1 mM IPTG was added, the culture medium was cultivated for an additional 4 hours. The cells were harvested from each of the four transformants to measure their enzyme activities and subject them to the reduction reaction of ethyl 4-chloroacetoacetate.

EXAMPLE 23

Enzyme Activities of *E. coli* Strains Transformed with pSG-KAR1

The grown cells (1.5 mL of the culture) of *E. coli* transformed with plasmid pSG-KAR1 prepared in Example 22 were disrupted by the method described in Example 16, and the cell extract was prepared to assay its enzyme activities. The enzymatic reaction for assaying the glucose dehydrogenase activity was performed at 25" in a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 2.5 mM NAD^+, 100 mM D-glucose, and the enzyme. One unit of the enzyme is defined as the amount capable of catalyzing the formation of 1 "mol NADH in 1 min under conditions described above. Each enzyme activity of the crude enzyme solution from each recombinant *E. coli* is shown in Table 4.

TABLE 4

Enzyme activities of *E. coli* transformed with pSG-KAR1

	Enzyme activity	
Host	Glc-DH	ECAA-R
JM109	2.19	3.66
HB101	3.42	2.70
TG1	3.51	2.81
W3110	3.44	3.82

Enzyme activity: enzyme activity/1 ml culture
Glc-DH: glucose dehydrogenase activity
ECAA-R: reductase activity to ethyl 4-chloroacetoactate

EXAMPLE 24

Synthesis of ethyl(S)-4-chloro-3-hydorxybutyrate using *E. coli* Transformed with pSG-KAR1

E. coli HB101, TG1, and W3110 transformed with plasmid pSG-KAR1 prepared in Example 23 were cultured in LB liquid medium at 30" overnight. Each preculture was inoculated into 2×YT medium (Bacto-Tryptone 20 g, Bacto-Yeast extract 10 g, NaCl 10 g/L) and grown at 37" for 4 hours. After 0.1 mM IPTG was added, the cultured medium was cultivated for an additional 4 hours. The grown cells were collected, and their reductase activity to ethyl 4-chloroacetoacetate was measured.

The enzyme reaction was performed at 25" overnight with shaking in 20 ml of a reaction mixture containing the *E. coli* cells prepared from 20 ml of the cultured medium, 200 mM potassium phosphate buffer (pH 6.5), 5% ethyl 4-chloroacetoacetate, 607 mM D-glucose, and 1 mM NAD^+. Separately, the reaction was performed under the same conditions described above but without NAD^+. The amount of ethyl(S)-4-chloro-3-hydorxybutyrate and its purity were determined in the same manner described in Example 9, and the results are shown in Table 5.

TABLE 5

Synthesis of SECHB using *E. coli* transformed with pSG-KAR1

	NAD^+ added		NAD^+ not added	
Host	(g/L)	% ee(s)	(g/L)	% ee(s)
HB101	42.3	99.0	13.2	99.9
TG1	42.5	99.4	19.3	99.9
W3110	49.9	99.9	23.0	99.9

SECHB: ethyl (S)-4-chloro-3-hydroxybutyrate

SEQUENCE LISTING

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ttgacagtta tcactggtgg agcaggagcc attggcggag ctctgtgtga gggatttgcg 180

tcctgtggat ctgacgttgt cattttagat tacaaataca gtcctgaatt gtcatcagtt 240

ttggaatcta ggtatggagt gaggtcgaaa agctatcagg tcgacattac gagttcagaa 300

gacgtgaaac ttgttgttgc aaagatttta gaagattttc ctgatcgcga tatcaataca 360

tttgttgcta atgcaggtat tgcatggacc aacggttcca ttttgaacga aaacgcgacg 420

ccagatgtgt ggaaacgtgt tatggatgtg aacgtgcaag gaacttatca ttgtgcgaaa 480

tatgtggcag aagtgttcaa acaacagggc catggtaatc tgattttgac tgcgtcgatg 540

tcaagttata taagcaacgt tcccaactac caaacatgtt ataatgcctc taaagcggcc 600

gtcagacata tggcaaaggg atttgctgtt gaattcgccc atttgacaaa ccccgcaggt 660

aaaatcagat gcaattcggt ttcacctggt tacactgaca ccgcactttc agcttttgtt 720

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caagagctag tcggagccta cttgtatttg gcatctgacg ctgcatcatt cacaaatgga 840

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<210> SEQ ID NO 2
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<212> TYPE: PRT
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Pro Gln Glu Pro Pro Lys Glu Gln Tyr Pro Asp Gly Val Asn Tyr Leu
20 25 30

Ser Leu Phe Ser Gln Lys Gly Lys Leu Thr Val Ile Thr Gly Gly Ala
35 40 45

Gly Ala Ile Gly Gly Ala Leu Cys Glu Gly Phe Ala Ser Cys Gly Ser
50 55 60

Asp Val Val Ile Leu Asp Tyr Lys Tyr Ser Pro Glu Leu Ser Ser Val
65 70 75 80

Leu Glu Ser Arg Tyr Gly Val Arg Ser Lys Ser Tyr Gln Val Asp Ile
85 90 95

Thr Ser Ser Glu Asp Val Lys Leu Val Val Ala Lys Ile Leu Glu Asp
100 105 110

Phe Pro Asp Arg Asp Ile Asn Thr Phe Val Ala Asn Ala Gly Ile Ala
115 120 125

Trp Thr Asn Gly Ser Ile Leu Asn Glu Asn Ala Thr Pro Asp Val Trp
130 135 140

Lys Arg Val Met Asp Val Asn Val Gln Gly Thr Tyr His Cys Ala Lys
145 150 155 160

Tyr Val Ala Glu Val Phe Lys Gln Gln Gly His Gly Asn Leu Ile Leu
165 170 175

Thr Ala Ser Met Ser Ser Tyr Ile Ser Asn Val Pro Asn Tyr Gln Thr
180 185 190

Cys Tyr Asn Ala Ser Lys Ala Ala Val Arg His Met Ala Lys Gly Phe

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Ala Val Glu Phe Ala His Leu Thr Asn Pro Ala Gly Lys Ile Arg Cys
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Asn Ser Val Ser Pro Gly Tyr Thr Asp Thr Ala Leu Ser Ala Phe Val
225                 230                 235                 240

Pro Val Glu Gln Arg Ala Gln Trp Trp Gly Leu Thr Pro Met Gly Arg
                245                 250                 255

Glu Ala Leu Pro Gln Glu Leu Val Gly Ala Tyr Leu Tyr Leu Ala Ser
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Asp Ala Ala Ser Phe Thr Asn Gly Cys Asp Ile Gln Val Asp Gly Gly
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Tyr Thr Cys Val
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<223> OTHER INFORMATION: n = A, T, G, or C

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ccaccgaagg agcaatatcc cgatggtgtt aattacttga gcttgttcag tcagaaaggg 120

aaattgacag ttatcactgg tggagcagga gccattggcg gagctctgtg tgagggattt 180

gcgtcctgtg gatctgacgt tgtcatttta gattacaaat actctcctga attatcttct 240

gtattagaaa gctt 254

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<212> TYPE: DNA
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ccaccgaagg agcaatatcc cgatggtgtt aattacttga gcttgttcag tcagaaaggg 120

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aaatatgtgg cagaagtgtt caaacaacag ggccatggta atctgatttt gactgcgtcg 540

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gaagattttc ctgatcgcga tatcaataca tttgttgcta atgcaggtat tgcatggacc 180

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aacgtgcaag gaacttatca ttgtgcgaaa tatgtggcag aagtgttcaa acaacagggc 300

catggtaatc tgattttgac tgcgtcgatg tcaagttata taagcaacgt tcccaactac 360

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<220> FEATURE:
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<400> SEQUENCE: 15

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<210> SEQ ID NO 16
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 1787
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maagacaagg cgraatagat gacgracgtt ggctgtaaat gtcgggggac naaatagatg 180

caaatawtws wgmnaymwww gkmmymkwyn ttttttaaat agcctggtta actacggcag 240

catgggctcg gtggtaggga aagaacaatt agtctatatt taggagagag gtataaataa 300

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catgaactct ttgatatgta tcttatgtta ttttttcttt aagcgacttc atagtggttt 420

cgggcctcgt tcatcggaga gctagctttg cacctgagtt tgggtttaga cacactataa 480

gaagagttta aagtctagga agtattcaaa aaataaagta aaagtcgcaa tgacgtttca 540

gcatttttta agaggtggat tagaagataa aacagttcct caggagccac cgaaggagca 600

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cactggtgga gcaggagcca ttggcggagc tctgtgtgag ggatttgcgt cctgtggatc 720

tgacgttgtc attttagatt acaaatacag tcctgaattg tcatcagttt tggaatctag 780

gtatggagtg aggtcgaaaa gctatcaggt cgacattacg agttcagaag acgtgaaact 840

tgttgttgca aagattttag aagattttcc tgatcgcgat atcaatacat ttgttgctaa 900

tgcaggtatt gcatggacca acggttccat tttgaacgaa aacgcgacgc cagatgtgtg 960

gaaacgtgtt atggatgtga acgtgcaagg aacttatcat tgtgcgaaat atgtggcaga 1020

agtgttcaaa caacagggcc atggtaatct gattttgact gcgtcgatgt caagttatat 1080

aagcaacgtt cccaactacc aaacatgtta taatgcctct aaagcggccg tcagacatat 1140

ggcaaaggga tttgctgttg aattcgccca tttgacaaac cccgcaggta aaatcagatg 1200

caattcggtt tcacctggtt acactgacac cgcactttca gcttttgttc cggtcgaaca 1260

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cgttatanaa tttggctama agtwttanca natnyancga attattcama rawmwtttyc 1500

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aaccccgcag gtaaaatcag atgcaattcg gtttcacctg gttacactga caccgcactt 720

tcagcttttg ttccggtcga acagcgcgct cagtggtggg gattgactcc tatgggtcgc 780

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    50                  55                  60

Ser Cys Gly Ser Asp Val Val Ile Leu Asp Tyr Lys Tyr Ser Pro Glu
65                  70                  75                  80

Leu Ser Ser Val Leu Glu Ser Arg Tyr Gly Val Arg Ser Lys Ser Tyr
                85                  90                  95

Gln Val Asp Ile Thr Ser Ser Glu Asp Val Lys Leu Val Val Ala Lys
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Ile Leu Glu Asp Phe Pro Asp Arg Asp Ile Asn Thr Phe Val Ala Asn
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His Cys Ala Lys Tyr Val Ala Glu Val Phe Lys Gln Gln Gly His Gly
                165                 170                 175

Asn Leu Ile Leu Thr Ala Ser Met Ser Ser Tyr Ile Ser Asn Val Pro
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Asn Tyr Gln Thr Cys Tyr Asn Ala Ser Lys Ala Ala Val Arg His Met
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Ala Lys Gly Phe Ala Val Glu Phe Ala His Leu Thr Asn Pro Ala Gly
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Lys Ile Arg Cys Asn Ser Val Ser Pro Gly Tyr Thr Asp Thr Ala Leu
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Ser Ala Phe Val Pro Val Glu Gln Arg Ala Gln Trp Trp Gly Leu Thr
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aataaacaag atccgaacga ggtaaaagaa gaggtcatca aggcgggcgg tgaagctgtt 180

gtcgtccaag gagatgtcac gaaagaggaa gatgtaaaaa atatcgtgca aacggcaatt 240

aaggagttcg gcacactcga tattatgatt aataatgccg gtcttgaaaa tcctgtgcca 300

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agtaaaggcg ggataaagct gatgacagaa acattagcgt tggaatacgc gccgaagggc 540

attcgcgtca ataatattgg gccaggtgcg atcaacacgc caatcaatgc tgaaaaattc 600

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35 40 45

Lys Glu Glu Val Ile Lys Ala Gly Gly Glu Ala Val Val Val Gln Gly
50 55 60

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Asp Val Thr Lys Glu Glu Asp Val Lys Asn Ile Val Gln Thr Ala Ile
 65                  70                  75                  80

Lys Glu Phe Gly Thr Leu Asp Ile Met Ile Asn Asn Ala Gly Leu Glu
                85                  90                  95

Asn Pro Val Pro Ser His Glu Met Pro Leu Lys Asp Trp Asp Lys Val
            100                 105                 110

Ile Gly Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg Glu Ala Ile
        115                 120                 125

Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Asn Val Ile Asn Met Ser
    130                 135                 140

Ser Val His Glu Val Ile Pro Trp Pro Leu Phe Val His Tyr Ala Ala
145                 150                 155                 160

Ser Lys Gly Gly Ile Lys Leu Met Thr Glu Thr Leu Ala Leu Glu Tyr
                165                 170                 175

Ala Pro Lys Gly Ile Arg Val Asn Asn Ile Gly Pro Gly Ala Ile Asn
            180                 185                 190

Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Lys Gln Lys Ala Asp
        195                 200                 205

Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Glu Pro Glu Glu Ile
    210                 215                 220

Ala Ala Val Ala Ala Trp Leu Ala Ser Lys Glu Ala Ser Tyr Val Thr
225                 230                 235                 240

Gly Ile Thr Leu Phe Ala Asp Gly Gly Met Thr Gln Tyr Pro Ser Phe
                245                 250                 255

Gln Ala Gly Arg Gly
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```

What is claimed is:

1. A purified carbonyl reductase having the following physicochemical properties:

reduces 4-haloacetoacetate ester to produce (S)-4-halo-3-hydroxybutyrate ester using reduced β-nicotinamide ademne dinucleotide as an electron donor;

has high reductase activity for 4-chloroacetoacetate ester but does not substantially dehydrogenate any optical isomers of 4-halo-3-hydroxybutyrate ester;

shows higher enzymatic activity when used with reduced β-nicotinamide adenine dinucleotide as an electron donor than when used with reduced β-nicotinamide adenine dinucleotide phosphate;

has an optimal pH of 5.0 to 6.0;

does not substantially dehydrogenate isopropanol and does not reduce acetoacetate; and

has a molecular weight of about 32,000 daltons when determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis;

wherein the carbonyl reductase is a *Kluyveromyces aestuarii* carbonyl reductase.

* * * * *

The Journal of Experimental Biology 207, 4095-4104
Published by The Company of Biologists 2004
doi:10.1242/jeb.01252

Presence and properties of cellulase and hemicellulase enzymes of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes*

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Accepted 18 August 2004

Summary

Digestive juice from the herbivorous gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes* exhibited total cellulase activity and activities of two cellulase enzymes; endo-β-1,4-glucanase and β-1,4-glucosidase. These enzymes hydrolysed native cellulose to glucose. The digestive juice of both species also contained laminarinase, licheninase and xylanase, which hydrolysed laminarin, lichenin and xylan, respectively, to component sugars. The pH optima of β-1,4-glucosidase, endo-β-1,4-glucanase and total cellulase from *G. natalis* were 4–5.5, 5.5 and 5.5–7, respectively. In the digestive juice from *D. hirtipes*, the corresponding values were 4–7, 5.5–7 and 4–9, respectively. The pH of the digestive juice was 6.69±0.03 for *G. natalis* and 6.03±0.04 for *D. hirtipes* and it is likely that the cellulases operate near maximally *in vivo*. In *G. natalis*, total cellulase activity and endo-β-1,4-glucanase activity were higher than in *D. hirtipes*, and the former species can thus hydrolyse cellulose more rapidly. β-1,4-glucosidase from *G. natalis* was inhibited less by gluconoD-lactone (K_i=11.12 mmol l^{-1}) than was the β-1,4-glucosidase from *D. hirtipes* (K_i=4.53 mmol l^{-1}). The greater resistance to inhibition by the β-1,4-glucosidase from *G. natalis* may contribute to the efficiency of the cellulase system *in vivo* by counteracting the effects of product inhibition and possibly dietary tannins. The activity of β-1,4-glucosidase in the digestive juice of *D. hirtipes* was higher than that of *G. natalis*.

Key words: land crab, *Gecarcoidea natalis*, *Discoplax hirtipes*, cellulase, endo-β-1,4-glucanase, cellobiohydrolase, β-1,4-glucosidase, laminarinase, xylanase, licheninase, fibre digestion.

Introduction

Plant material contains significant amounts of structural polysaccharides, particularly cellulose and a range of hemicelluloses. These polymers are comprised of sugar units, usually glucose, joined by β-1,4 and β-1,3 glycosidic bonds. Whilst vertebrates generally lack the enzymes necessary to break these bonds, and can only access the sugars *via* the activity of microorganisms in the alimentary canal, cellulose digestion is common amongst invertebrates, and an increasing number have been shown to possess their own cellulases (Watanabe and Tokuda, 2001).

Crystalline or native cellulose is hydrolysed to its component glucose units by the combined activities of endo-β-1,4-glucanase (EG), cellobiohydrolase (CBH) and β-1,4-glucosidase. Endo-β,1-4-glucanase randomly hydrolyses internal β-1,4-glycosidic bonds of cellulose polymers of four or more glucose units (Scrivener and Slaytor, 1994; Tokuda et al., 1997; Wantanabe et al., 1997). It thus shortens cellulose chains, solubilizes cellulose polymers and provides a substrate for exo-β,1-4-glucosidase. However, it can also catalyse transglycosylation to rejoin the glucose oligomers (Lindner et al., 1983). Cellobiohydrolase attacks the non-reducing ends of cellulose chains and typically cleaves off the glucose-β-1,4 dimer, cellobiose. Cellobiohydrolase is also thought to disrupt the hydrogen bonding in crystalline cellulose, thus allowing the EG to endo-depolymerize. As this enzyme only attacks the ends of chains, its activity will be low unless endoglucanase is present to provide sufficient substrate. In the cockroach *Panesthia cribrata* and the termite *Reticulitermes speratus*, CBH activity is also catalyzed by the EG (Scrivener and Slaytor, 1994; Watanabe et al., 1997). The third enzyme involved in the complete hydrolysis of cellulose to glucose is β-1,4-glucosidase; this enzyme hydrolyses cello-oligosaccharides to glucose.

The term hemicellulose includes a range of alkali-soluble polysaccharides (Bacic et al., 1988). More specifically, they are defined as carbohydrate polymers of either xylose, glucose, mannose or mannose and glucose joined mainly by β-1,4 and β-1,3 glycosidic bonds (Bacic et al., 1988). Xylan, lichenin, β-D-glucan and laminarin are common hemicelluloses. Xylan, the next most abundant carbohydrate polymer after cellulose, is a β-1,4-linked polymer of xylose (Puls et al., 1988). Lichenin is a glucose polymer with the glucose units being joined with mainly β-1,4 glycosidic bonds and some β-1,3 glycosidic bonds in the chain (Bacic et al., 1988). Similar

polysaccharides, called mixed linkage β-glucan, are found in cereals and grasses (Terra and Ferreira, 1994). Like lichenin, laminarin (or callose) is a glucose polymer but with the sugars joined principally by β-1,3 glycosidic bonds (Bacic et al., 1988; Terra and Ferreira, 1994). Laminarin is present in the cell walls of fungi, in phloem and in plant wound tissue (Terra and Ferreira, 1994). Laminarin is the chief food reserve of algae (Vonk and Western, 1984). Xylanase, licheninase and laminarinase hydrolyse xylan, lichenin and laminarin, respectively (reviewed by Terra and Ferreira, 1994).

The diet of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes* mainly consists of green and brown leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998) of which cellulose and the hemicelluloses are major constituents. Cellulose makes up 12–18% of the dry matter of brown leaves of the fig (*Ficus macrophylla*) and 11% of the dry matter of green leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998), while hemicelluloses constitute 18–26% of the dry matter of brown fig leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). Both *G. natalis* and *D. hirtipes* assimilate substantial amounts of cellulose and hemicellulose from their leaf litter diet (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998); 43% and 49%, respectively, by *G. natalis* and 21% and 20%, respectively, by *D. hirtipes* fed brown leaf litter of *F. macrophylla* (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). As the crabs clearly hydrolyse these fibre components, it is probable that they possess cellulase and hemicellulase enzymes. In the present study, we investigated the presence and characteristics of cellulases and hemicellulases from *G. natalis* and *D. hirtipes*. Total cellulase activity and activities of β-1,4-glucosidase, EG, licheninase, laminarinase and xylanase were measured within the digestive juice of both species. Where possible, the kinetics (V_{max} and K_m) of the cellulase enzymes and the inhibitory constant (K_i) of glucono-D-lactone on β-1,4-glucosidase were also determined. It was envisaged that differences in the activities of the cellulase enzymes may explain reported differences in the assimilation of cellulose and hemicellulose between these two species.

Materials and methods

Materials

Gecarcoidea natalis (Pocock 1888) and *Discoplax hirtipes* (Dana 1851) were collected from rainforest in the Australian Territory of Christmas Island, Indian Ocean and airfreighted to Sydney where they were maintained at 25°C on a 12 h:12 h light:dark cycle. Tapwater was provided for drinking and the crabs were fed with fallen leaves of *Ficus macrophylla* Desf. ex Pers. subspecies *macrophylla*.

Methods

Digestive juice used in measurements was taken from the foreguts of experimental animals as follows. The crabs were held ventral side up on a polystyrene board, and a fine polythene tube was inserted into the cardiac stomach *via* the mouth and oesophagus. A small plastic wedge was used to prevent the mandibles from cutting the tube. Up to 2 ml of dark brown digestive juice could be collected by gentle suction with a 2-ml syringe attached to the tubing. The procedure did not harm the crabs. Fluid was centrifuged at 10 000 ***g*** for 5 min, to remove food debris, and the supernatant was used for analyses. Fluid could be stored at 4°C for several days without loss of enzyme activity.

Measurement of enzyme activities

Cellulase activities

Total cellulase activity and activities of β-1,4-glucosidase (cellobiase; EC 3.2.1.21) and EG (EC 3.2.1.4) were measured in digestive juice taken from the cardiac stomach of *Gecarcoidea natalis* and *Discoplax hirtipes* using modified versions of the methods of Schulz et al. (1986) and Hogan et al. (1988). Reactions and incubations were carried out at 40°C in 1.5-ml Eppendorf centrifuge tubes in an Eppendorf thermomixer. Measurement at 40°C allowed direct comparison with data for cellulase activities of other invertebrates. Absorption values of the samples were measured using an LKB Ultraspec II spectrophotometer. Activities of the enzymes are presented per ml of digestive juice. Expression per mg of protein is not meaningful in this situation where crude juice is used since the bulk of the protein does not represent the enzyme of interest, is highly variable and may even be dietary in origin. It is likely that the volume of fluid in the foregut remains relatively constant. Hanes plots derived from the data on cellulase activity at different substrate concentrations were used to determine if enzyme activity followed Michaelis-Menten kinetics. Where this was established, the kinetic parameters (K_m, V_{max}) were then calculated from the plots.

β-1,4-glucosidase. Activity was measured as the rate of production of glucose from cellobiose (Cat. No. C-7252; Sigma Chemical Corp., St Louis, MO, USA). Digestive juice (25 μl) was mixed with 25 μl of 0.1 mol l^{-1} acetate buffer (pH 5.5) and 50 μl of either 2.92, 14.61, 29.21 or 58.4 mmol l^{-1} cellobiose in the same buffer, and the mixture was incubated at 40°C for 30 min. The reaction was stopped by the addition of 25 μl of 0.3 mol l^{-1} tri-chloro acetic acid, and excess acid was neutralized with 5 μl of 2.5 mol l^{-1} K_2CO_3. Precipitated protein was pelleted by centrifugation at 10 000 ***g*** for 10 min. A blank (75 μl buffer plus 25 μl digestive juice) and a standard (50 μl of 7 mol l^{-1} glucose in buffer + 25 μl digestive juice + 25 μl buffer) were prepared for each sample analysed. This enabled correction for the background absorption due to the digestive juice at the wavelength measured.

Glucose concentration was measured in 50 μl (*G. natalis*) or 25 μl (*D. hirtipes*) samples of the incubation mixture using a commercial glucose assay kit (Sigma Cat No. 510-A). The 50 μl or 25 μl samples were diluted to a total of 100 μl with water in a 1.5-ml micro test tube. 1 ml of the colour reagent supplied with the kit was then added, and the mixture was vortexed and incubated at 37°C for 30 min. After incubation, the absorbance of the samples was read at 445 nm.

Glucono-D-lactone competitively inhibits β-1,4-glucosidase (Scrivener and Slaytor, 1994; Shewale and Sadana, 1981; Santos and Terra, 1985). The inhibitory constant of glucono-D-lactone on β-1,4-glucosidase was also determined by measuring β-1,4-glucosidase activity in the presence of 20 mmol l^{-1} glucono-D-lactone and 0–58.43 mmol l^{-1} cellobiose.

Endo-β-1,4-glucanase. Activity was measured as the rate of production of reducing sugars from the substrate, carboxymethyl cellulose (Sigma Cat. No. C-5678). Digestive juice (20 μl) was mixed with 80 μl buffer and 100 μl of 0.1, 0.5, 1 and 2% (w/v) carboxymethyl cellulose in the same buffer. Blanks contained 20 μl of digestive juice and 180 μl of buffer, while standards contained 20 μl of digestive juice plus 100 μl glucose (13 mmol l^{-1}) in buffer and 80 μl buffer. The buffer was 0.1 mol l^{-1} acetate buffer, pH 5.5, containing 30 mmol l^{-1} of the β-1,4-glucosidase inhibitor glucono-D-lactone. Samples, standards and blanks were incubated at 40°C for 10 min and the reaction stopped by the addition of 25 μl of 0.3 mol l^{-1} HCl. Excess acid was then neutralized by the addition of 5 μl of 2.5 mol l^{-1} K_2CO_3. The reducing sugar produced during the incubation was measured as glucose equivalents by the tetrazolium blue method of Jue and Lipke (1985) using 5 mmol l^{-1} glucose as a standard. Absorption of samples, standards and blanks was read at 660 nm.

Total cellulase activity. Total cellulase activity was measured as the rate of production of glucose from microcrystalline cellulose (Sigmacell 20). Digestive juice (50·μl) was mixed with 100 μl of either 0.1, 0.5, 1 or 2% (w/v) Sigmacell 20 (Sigma Cat. No. S3504) made up in buffer. Suspension of the cellulose was ensured by vortexing the stock cellulose immediately before pipetting. Blanks contained digestive juice and buffer while standards contained digestive juice, buffer and 7 mmol l^{-1} glucose. The buffer used was 0.1 mol l^{-1} acetate, pH 5.5. The mixture was incubated and agitated for 60 min at 40°C in an Eppendorf thermomixer before the reaction was stopped by the addition of 25 μl of 0.3 mol l^{-1} tri-chloro acetic acid. The excess acid was neutralized with 5 μl of 2.5 mol l^{-1} K_2CO_3 before assay of glucose. The incubation mixture was centrifuged (10 000 ***g*** for 10 min) and the glucose concentration determined in a 25 μl or 50 μl aliquot of the supernatant as described for β-1,4-glucosidase.

The K_m values for EG and CBH are given as mg substrate ml^{-1} reaction mixture since the substrates (carboxymethyl cellulose and cellulose) consist of carbohydrate polymers of varying length and do not have a uniform molecular mass.

Hemicellulase activities

Activities of the hemicellulase enzymes laminarinase [endo-β-1,3-glucanase (EC 3.2.1.39)], licheninase [endo-β-1,3; 1,4 glucanase (EC 3.2.1.73)], xylanase [endo-β-1,4-xylanase (EC 3.2.1.8) and 1,4-β-D-xylan xylanhydrolase (EC 3.2.1.37)] were measured in the digestive juice from *D. hirtipes* and *G. natalis*.

Laminarinase. Laminarinase activity was measured as the production of reducing sugars from the hydrolysis of laminarin (from *Laminaria digitata*; Sigma Cat. No. L-9634). Digestive juice (20 μl) was mixed with 50 μl of 1% (w/v) laminarin and 130 μl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. Blanks and standards were run at the same time. Blanks consisted of 20 μl of digestive juice and 180 μl of assay buffer, while standards consisted of 20 μl of digestive juice, 100 μl of 13 mmol l^{-1} glucose and 80 μl of assay buffer. Samples, blanks and standards were incubated with agitation at 40°C for 10 min. The reaction was stopped by the addition of 50 μl of 0.3 mol l^{-1} HCl and neutralized with 10 μl of 2.5 mol l^{-1} K_2CO_3. Reducing sugars were measured in a 10 μl aliquot as described above.

Licheninase. The activity of licheninase was measured by the production of reducing sugars from the hydrolysis of lichenin (from *Cetraria islandica*; Sigma Cat. No. L-6133). Digestive juice (20 μl) was mixed with 100 μl of 0.1% (w/v) lichenin and 80 μl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. To correct for the background absorbance of the digestive juice, blanks and standards were run at the same time. Digestive juice (20 μl) and 180 μl of assay buffer constituted the blank while 20 μl of digestive juice plus 100 μl of 13 mmol l^{-1} glucose and 80 μl of assay buffer constituted the standard. Samples, blanks and standards were incubated with agitation at 40°C for 10 min. The reaction was stopped with 50 μl of 0.3 mol l^{-1} HCl and the mixture was then neutralized with 10 μl of 2.5 mol l^{-1} K_2CO_3. Reducing sugars were measured in a 10 μl sample as described above.

Xylanase. Xylanase activity was measured as the production of reducing sugars from the hydrolysis of xylan (from birchwood, *Betula*; Sigma Cat. No. X-0502). Digestive juice (20·μl) was incubated with 100 μl of 1% (w/v) xylan and 80 μl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. Blanks (20 μl of digestive juice and 180 μl of buffer) and standards (20 μl of digestive juice, 100 μl of 13 mmol l^{-1} glucose and 80 μl of assay buffer) were run at the same time. Samples, blanks and standards were incubated with agitation at 40°C for 60 min. After this period, the reaction was stopped by precipitating the protein with 50 μl of 0.3 mol l^{-1} HCl and was neutralized by the addition of 10 μl of 2.5 mol l^{-1} K_2CO_3. Reducing sugars were measured in a 10 μl sample of this reaction mixture as described above.

pH

The activities of β-1,4-glucosidase, EG and total cellulase were measured over a pH range of 4–9. Acetate buffer was used to maintain pH values of 4 and 5.5, and Tris buffer was used for the higher pH values of 7–9. The pH of gut fluid was measured anaerobically at 25°C using freshly drawn samples of fluid from the foregut and a Radiometer G299a capillary pH electrode and Radiometer PHM 73 meter (Radiometer, Copenhagen, Denmark).

Protein

The concentrations of protein in samples of digestive juice were measured using a BioRad protein assay kit and bovine γ-globulin standard (BioRad, Hercules, CA, USA).

Statistics

Statistical comparisons (ANCOVA and one- and two-way ANOVA with Tukey's HSD *post hoc* tests) were made using the statistical computing package Systat 7 (Systat Software Inc., Richmond, CA, USA) to calculate the statistical probabilities.

Results

Cellulase activity in digestive juice

Incubation of crystalline cellulose with digestive juice from the foregut of *G. natalis* and *D. hirtipes* yielded glucose and thus established the presence of total cellulase activity (Fig. 1). As such activity is likely to result from the combined activity of CBH, EG and β-1,4-glucosidase, assays were then conducted to test for activity of each enzyme (Figs 1–3).

The presence of EG was confirmed by the production of reducing sugars during the incubation of carboxymethyl cellulose with digestive juice (Fig. 2). Carboxymethyl cellulose is not a substrate for either CBH or β-1,4-glucosidase. In similar fashion, the presence of β-1,4-glucosidase in digestive juice was confirmed by the production of significant quantities of glucose when cellobiose was incubated with digestive juice (Fig. 3). This enzyme is generally ineffective against crystalline or carboxymethyl cellulose. The activities of total cellulase, EG and β-1,4-glucosidase, from both *G. natalis* and *D. hirtipes*, initially increased with substrate concentration but levelled out at high concentrations (Figs 1–3), consistent with saturation of these enzymes at the higher substrate concentrations.

pH and cellulase activities

Foregut fluid from both study species was slightly acidic, with that of *D. hirtipes* (6.03±0.04, N=7) significantly more acid than the juice from *G. natalis* (6.69±0.03, N=4) (t-test; P<0.001). The activities of β-1,4-glucosidase, EG and total cellulase from both *G. natalis* and *D. hirtipes* were affected by the pH of the incubation buffer (Table 1) and shared similar pH maxima and hence pH ranges for optimal activity. For total cellulase from *G. natalis*, optimal activity occurred at pH 5.5, but for *D. hirtipes* total cellulase activities were low with no obvious pH optimum (Table 1). For β-1,4-glucosidase, optimal activity occurred between pH 4 and 7 (Table 1); high levels of activity were measured at both pH 4 and pH 5.5 for *G. natalis* and at pH 4, 5.5 and 7 for *D. hirtipes* (Table 1). Endo-β-1,4-glucanase activities were maximal at pH 5.5, 7 and 9 and lower at pH 4 and 8 in digestive juice from both *D. hirtipes* and *G. natalis* (Table 1).

Enzyme activities

Total cellulase activity

The total cellulase activity on crystalline cellulose was higher in the digestive juice from *G. natalis* than in that from *D. hirtipes* (Fig. 1); 2.8× higher at the highest substrate concentration used of 13.3 mg ml^{-1} cellulose (Table 2). Total cellulase activity from *G. natalis* followed Michaelis-Menten kinetics since the Hanes plot of its activity was linear (r^2=0.624, P=0.001; Fig. 4). Total cellulase activity had a K_m of 2.43 mg ml^{-1} cellulose incubation mixture and a V_{max} of 0.117 μmol min^{-1} ml^{-1} digestive juice (Table 2). By contrast, the Hanes plot of total cellulase activity from *D. hirtipes* was

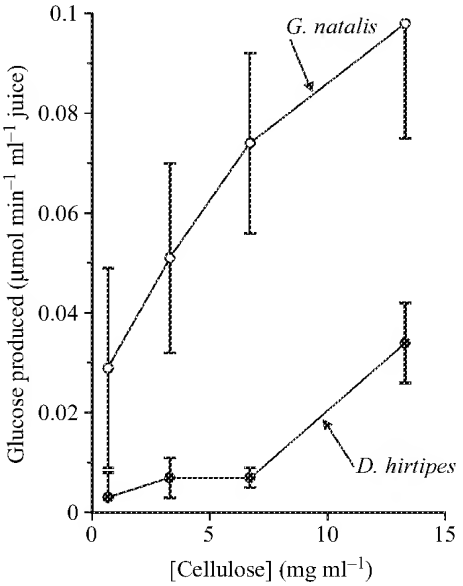

Fig. 1. Total cellulase activity in digestive juice from *G. natalis* and *D. hirtipes*. Activity differed significantly among cellulose concentrations (P<0.05) and between *G. natalis* and *D. hirtipes* (two-way ANOVA).

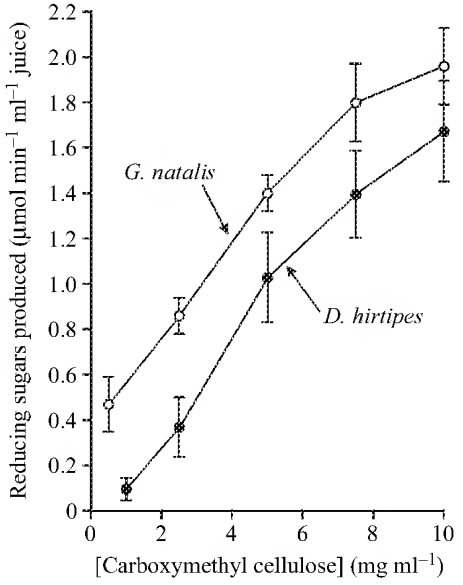

Fig. 2. Activities of endo-β-1,4-glucanase in digestive juice of *G. natalis* and *D. hirtipes*. Enzyme activities were significantly different (P<0.05) between concentrations of carboxymethyl cellulose and between species (two-way ANOVA).

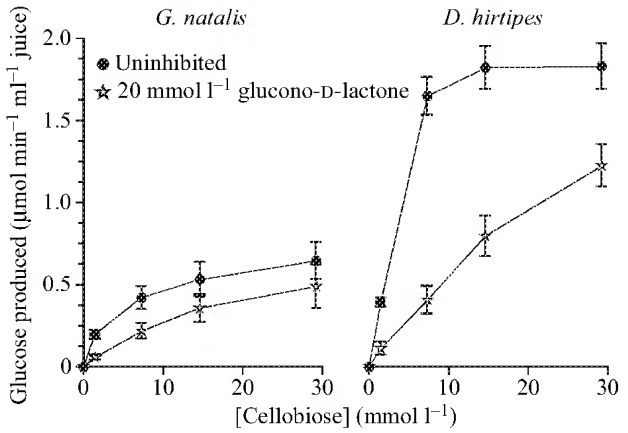

Fig. 3. Activities of β-1,4-glucosidase activities in digestive juice of *G. natalis* and *D. hirtipes* in the presence (stars) and absence (circles) of 20 mmol l^{-1} glucono-D-lactone. For both *G. natalis* and *D. hirtipes*, the β-1,4-glucosidase activity increased with increasing cellobiose concentrations and was higher in the absence of glucono-D-lactone ($P<0.05$; two-way ANOVA). For β-1,4-glucosidase activity from *D. hirtipes*, there was a significant interaction ($P<0.05$) between cellobiose concentration and treatment (two-way ANOVA). Uninhibited β-1,4-glucosidase activities from *D. hirtipes* were higher than uninhibited β-glucosidase activities from *G. natalis* ($P<0.05$; two-way ANOVA).

not linear ($r^2=0.003$, $P=0.774$; Fig. 4) and did not follow Michaelis-Menten kinetics (Fig. 4), so its kinetic parameters could not be calculated.

Endo-β-1,4-glucanase

Endo-β-1,4-glucanase activities in digestive juice were slightly, but consistently, higher in *G. natalis* than in *D. hirtipes* ($P<0.05$; two-way ANOVA; Fig. 2). The differences in activities between species were maintained across the range of substrate concentration used. Endo-β-1,4-glucanase activity from *G. natalis* was 1.17× the EG activity from *D. hirtipes* at a substrate concentration of 10 mg ml^{-1} CMC (Table 2). The Hanes plot of the EG activity from *G. natalis* was linear ($r^2=0.514$, $P=0.0001$) while that from *D. hirtipes* was not ($r^2=0.041$, $P=0.2$; Fig. 5). Hence, the EG from *G. natalis* followed Michaelis-Menten kinetics (Fig. 5) with a K_m of 3.03 mg ml^{-1} CMC incubation mixture and a V_{max} of 6.11 μmol min^{-1} ml^{-1} digestive juice (Table 2). Equivalent values could not be calculated for EG from *D. hirtipes*.

β-1,4-glucosidase

The activity of β-1,4-glucosidase from *D. hirtipes* was higher than that from *G. natalis* ($P<0.05$; two-way ANOVA; Fig. 3). At a cellobiose concentration of 29.22 mmol l^{-1}, the activities differed by a factor of 2.8 (Fig. 3; Table 2). β-1,4-Glucosidase from both species followed Michaelis-Menten kinetics since Hanes plots of β-glucosidase activities were linear (Fig. 6). The V_{max} of β-1,4-glucosidase from *D. hirtipes* was 3.4× higher than that from *G. natalis* ($P<0.005$; ANCOVA; Table 2) and the K_m value for β-1,4-glucosidase from *D. hirtipes* was lower than that from *G. natalis* ($P<0.001$; ANCOVA; Table 2). Hence, β-1,4-glucosidase activity from *D. hirtipes* saturated at lower concentrations of cellobiose and was higher at all substrate concentrations than β-1,4-glucosidase from *G. natalis*.

Effect of inhibitor on the activity of β-1,4-glucosidase

V_{max} values for inhibited and uninhibited β-glucosidase were similar in both *G. natalis* and *D. hirtipes* (Figs 3, 6; Table 2). The K_m of β-1,4-glucosidase in the presence of 20 mmol l^{-1} glucono-D-lactone was much higher than in its absence (Figs 3, 6; Table 2).

This situation (similar V_{max} but different K_m values in the presence and absence of an inhibitor) is indicative of competitive inhibition of β-1,4-glucosidase by glucono-D-

Table 1. *The effect of pH on the activity of cellulase enzymes from the foregut of the gecarcinid crabs* Gecarcoidea natalis *and* Discoplax hirtipes

	Activity (μmol min⁻¹ ml⁻¹ digestive juice)				
Enzyme	pH 4	pH 5.5	pH 7	pH 8	pH 9
Total cellulase					
G. natalis	0.018±0.016[a]	0.043±0.016[b]	0.017±0.010[a]	0.021±0.020[a]	0.011±0.011[a]
D. hirtipes	0.014±0.005[a]	0.020±0.005[a]	0.023±0.006[a]	0.005±0.002[a]	0.010±0.003[a]
β-1,4-glucosidase					
G. natalis	0.397±0.253[a,b]	0.521±0.253[a]	0.254±0.131[b,c]	0.075±0.032[c,d]	0.031±0.019[d]
D. hirtipes	1.779±0.083[a]	1.729±0.066[a]	1.961±0.074[a]	0.214±0.046[b]	0.121±0.028[b]
Endo-β-1,4-glucanase					
G. natalis	0.297±0.174[a]	2.076±0.104[b]	2.304±0.116[b]	0.945±0.189[c]	1.981±0.106[b]
D. hirtipes	0.627±0.092[a]	1.534±0.206[b]	1.434±0.101[b]	0.710±0.169[a]	1.041±0.151[a,b]

Total cellulase and β-1,4-glucosidase activities were measured as the rate of glucose production from the hydrolysis of either crystalline cellulose or cellobiose, respectively. Endo-β-1,4-glucanase activities were measured as the rate of reducing sugars produced from the hydrolysis of carboxymethyl cellulose. Within a row, similar superscript letters indicate similar means ($P>0.05$). Values are means ± S.E.M. ($N=10$).

Table 2. *Kinetic parameters (K_m, V_{max}) and activities of total cellulose, endo-β-1,4-glucanase and β-1,4-glucosidase from the foregut fluid of* Gecarcoidea natalis *and* Discoplax hirtipes

	Gecarcoidea natalis			*Discoplax hirtipes*		
Enzyme	Activity (μmol min^{-1} ml^{-1})	K_m	V_{max} (μmol min^{-1} ml^{-1})	Activity (μmol min^{-1} ml^{-1})	K_m	V_{max} (μmol min^{-1} ml^{-1})
Total cellulase	0.01±0.23	2.43 mg ml^{-1} cellulose	0.117	0.03±0.01		
Endo-β-1,4-glucanase	1.96±0.17	3.03 mg ml^{-1} CMC	6.11	1.67±0.22		
β-1,4-glucosidase						
Uninhibited	0.655±0.11	5.84 mmol l^{-1} cellobiose[c]	0.606[a]	1.83±0.14	4.23 mmol l^{-1} cellobiose[f]	2.08[e]
Inhibited (20 mmol l^{-1} glucono-D-lactone)	0.49±0.13	16.28 mmol l^{-1} cellobiose[d]	0.721[a]	1.22±0.13	22.87 mmol l^{-1} cellobiose[g]	2.16[e]

Activities of total cellulase, endo-β-1,4-glucanase (EG) and β-1,4-glucosidase were measured at substrate concentrations of 13.3 mg ml^{-1} cellulose, 10 mg ml^{-1} carboxymethyl cellulose (CMC) and 29.22 mmol l^{-1} cellobiose, respectively. Enzyme activities are expressed as μmol glucose min^{-1} ml^{-1} digestive juice (total cellulase and β-1,4-glucosidase) or as μmol reducing sugars min^{-1} ml^{-1} (EG). Inhibited β-glucosidase from both *G. natalis* and *D. hirtipes* had a similar V_{max} and a significantly higher K_m than the uninhibited enzyme. Within a column, significant differences between values for β-glucosidase are indicated by different superscript letters. There were also significant differences in K_m and V_{max} between species for uninhibited β-1,4-glucosidase. Values are means ± S.E.M.

lactone in both species. Glucono-D-lactone has also been identified as a competitive inhibitor of β-1,4-glucosidase in previous studies (Scrivener and Slaytor, 1994; Shewale and Sadana, 1981; Santos and Terra, 1985). The inhibitory constant of glucono-D-lactone on β-1,4-glucosidase (K_i) was calculated using K_m values from Table 2 and the equation:

$$K_i = (K_m[I]) / (K_{app} - K_m) ,$$

where K_m is the K_m for β-1,4-glucosidase in the absence of glucono-D-lactone, K_{app} is the apparent K_m for β-1,4-glucosidase in the presence of 20 mmol l^{-1} glucono-D-lactone, and $[I]$ is the concentration of glucono-D-lactone used (20 mmol l^{-1}).

The K_i of glucono-D-lactone on β-1,4-glucosidase was 11.12 mmol l^{-1} for β-1,4-glucosidase from *G. natalis* and 4.53 mmol l^{-1} for β-1,4-glucosidase from *D. hirtipes*.

Hemicellulase activities in the digestive juice

Incubation of laminarin, lichenin or xylan with digestive juice from *G. natalis* or *D. hirtipes* yielded reducing sugars (Table 3), an outcome consistent with the presence of laminarinase, licheninase and xylanase in the digestive juice. The activities of the enzymes in the digestive juice of both *G. natalis* and *D. hirtipes* were in the order laminarinase > licheninase > xylanase (Table 3). Licheninase activity in the digestive juice from *D. hirtipes* was 1.73× that of *G. natalis* (Table 3) whilst the activities of laminarinase were similar between species and this was also the case for xylanase (Table 3).

Activities of the individual hemicellulases (licheninase, laminarinase and xylanase) measured were higher than those for total cellulase activity in the two species (Tables 2, 3). Laminarinase activity was much higher than the activity of EG and β-1,4-glucosidase (Tables 2, 3). For *G. natalis*, licheninase activity was similar to EG activity but higher than β-1,4-glucosidase activity (Tables 2, 3). Licheninase activity from *D. hirtipes* was higher than activities of both EG and β-1,4-glucosidase (Tables 2, 3). Xylanase activities for both *G. natalis* and *D. hirtipes* were lower than the respective EG and β-glucosidase activities (Tables 2, 3).

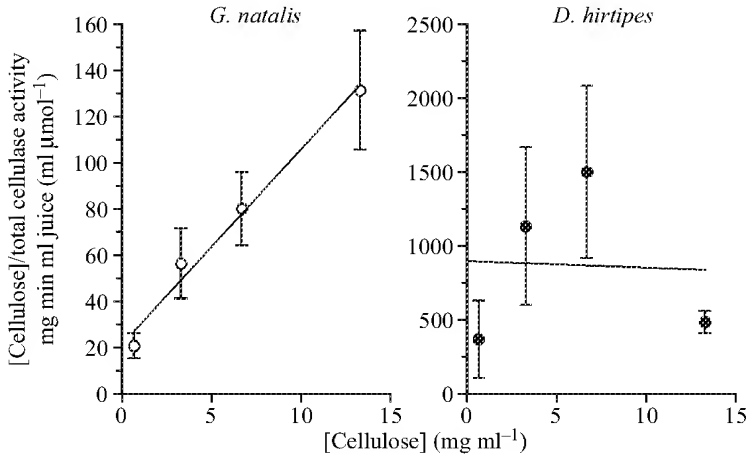

Fig. 4. Hanes plots of the activity of total cellulase activity from digestive juice of *G. natalis* and *D. hirtipes*. The plot was linear for *G. natalis* {[Cellulose]/total cellulase activity=8.52×[cellulose]+20.82 (r^2=0.624, P=0.001, N=7)} but not for *D. hirtipes* {[Cellulose]/total cellulase activity=952×[cellulose]–12.659 (r^2=0.003, P=0.774, N=10)}.

Discussion

Both *Gecarcoidea natalis* and *Discoplax hirtipes* digest significant amounts of the cellulose and hemicellulose present in their diets (Greenaway and

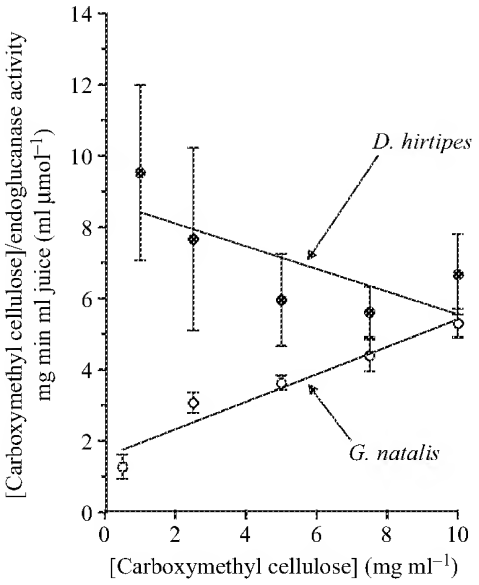

Fig. 5. Hanes plots of the activity of endo-β-1,4-glucanase activity from the digestive juice of *G. natalis* and *D. hirtipes*. The plot was linear for *G. natalis* {[CMC]/endo-β-1,4-glucanase activity= 0.329×[CMC]+2.018 (r^2=0.514, P=0.0001, N=7)} but not for *D. hirtipes* {[CMC]/endo-β-1,4-glucanase activity=–0.298×[CMC]+ 8.588 (r^2=0.041, P=0.2, N=10)}.

Linton, 1995; Greenaway and Raghaven, 1998) and in this study it has been demonstrated that this capacity is conferred by activity of cellulolytic and hemicellulolytic enzymes. The confirmed cellulase enzymes present were EG and β-1,4-glucosidase and the hemicellulases were laminarinase, licheninase and xylanase. Several invertebrates lack a separate CBH, and the CBH reaction is instead catalysed by EG (Scrivener and Slaytor, 1994; Watanabe et al., 1997). The presence of a separate CBH in the digestive juice of gecarcinid crabs remains to be established but, as in other animals, it may be absent.

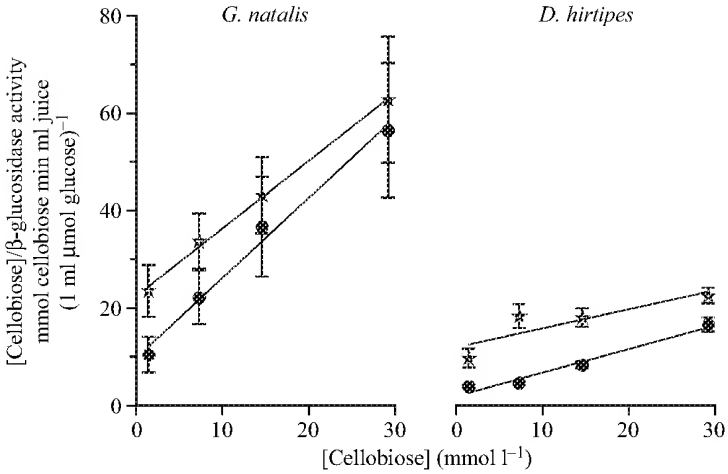

Fig. 6. Hanes plots of the activity data for β-1,4-glucosidase from the digestive juice of *G. natalis* and *D. hirtipes*. All plots were linear. *G. natalis*, uninhibited (circles) {[cellobiose]/β-1,4-glucosidase activity=1.651×[cellobiose]+9.650 (r^2=0.410, P=0.00, N=7)}; *G. natalis*, inhibited (stars) {[cellobiose]/β-1,4-glucosidase activity=1.387×[cellobiose]+22.58 (r^2=0.387, P=0.002, N=7)}. *D. hirtipes*, uninhibited (circles) {[cellobiose]/β-1,4-glucosidase activity=0.480×[cellobiose]+ 2.033 (r^2=0.832, P=0.00, N=7)}. *D. hirtipes*, inhibited (stars) {[cellobiose]/β-1,4-glucosidase activity=0.464×[cellobiose]+ 10.587 (r^2=0.450, P=0.000, N=7)}.

Table 3. *Activities of laminarinase, licheninase and xylanase in the digestive juice from* G. natalis *and* D. hirtipes *(µmol reducing sugars min^{-1} ml^{-1} digestive juice)*

	G. natalis	*D. hirtipes*
Laminarinase	12.09±0.49 (8)[a]	12.98±0.97 (8)[a]
Licheninase	1.76±0.19 (9)[a]	3.04±0.52 (7)[b]
Xylanase	0.33±0.05 (7)[a]	0.22±0.03 (8)[a]

Values are means ± S.E.M. (N). Similar superscript letters within a row indicate similar means (P>0.05).

Cellulase activity

Total cellulase and EG activities from *G. natalis* were higher than those from *D. hirtipes* (Figs 1, 2) and this should allow more rapid hydrolysis of cellulose by *G. natalis*, which is consistent with the earlier reports of higher cellulose assimilation efficiency in the latter species (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998).

In *G. natalis*, the K_m for EG was similar to values reported for other invertebrates (Tables 2, 4). The EG activity reported here may well exceed the *in vivo* value since the experimental substrate (carboxymethyl cellulose), unlike the natural substrate (crystalline cellulose), is in solution and more available for enzymatic hydrolysis.

β-1,4-glucosidase

β-1,4-glucosidase activity in the digestive juice exhibited a higher V_{max} and substrate affinity in *D. hirtipes* than in *G. natalis* (Fig. 3; Table 2). This contrasted with the findings for the other cellulase enzymes – EG and total cellulase activity – where activity was higher in *G. natalis*. This difference may be explained by the affinity of β-1,4-glucosidase for the inhibitor glucono-D-lactone and consequent substrate and product affinity (discussed below).

Competitive inhibition of β-1,4-glucosidase with glucono-D-lactone

The inhibition constant (K_i) for β-1,4-glucosidase was higher in *G. natalis* (11.12 mmol l^{-1}) than in *D. hirtipes* (4.53 mmol l^{-1}), and hence the β-1,4-glucosidase from *G. natalis* had a lower affinity for the inhibitor than that from *D. hirtipes*. This is consistent with the β-1,4-glucosidase from *G. natalis* being resistant to inhibition.

The high K_i of β-glucosidase from both gecarcinid species relative to K_i values for insects (Table 4) indicates that this enzyme is not particularly sensitive to inhibition by glucono-D-lactone in gecarcinids.

Glucono-D-lactone inhibits activity of β-1,4-glucosidase by mimicking the transitional form involved in the hydrolysis of the β-glycosidic bond (Terra and Ferreira, 1994). A lower affinity for the transitional form, and hence glucono-D-lactone, means that this form is not stabilized as well and the reaction would proceed at a slower rate, and this may explain why the digestive juice of *G. natalis* had a lower β-glucosidase activity than that from *D. hirtipes* (Fig. 3). Reduced β-1,4-glucosidase activity would not affect the overall rate of cellulose hydrolysis unless its activity was lower than that of the slowest step, probably the solubilization and initial depolymerization of the cellulose chains. A lower affinity for the transitional form would also mean that the affinity of the enzyme for its substrate and product would be reduced. This could account for lower substrate affinity exhibited by the β-1,4-glucosidase from *G. natalis* (cf. *D. hirtipes*; Fig. 3). In comparison with insects, the affinity of β-1,4-glucosidase from *G. natalis* was low since its K_m was in the upper range of the K_m for these animals (Tables 2, 4).

The inhibition resistance of β-1,4-glucosidase may counteract product inhibition. This means that the cellulase enzymes of *G. natalis* can hydrolyse cellulose despite the presence of the end product, glucose. Such a property would contribute to the overall efficiency of the cellulase system of *G. natalis*. The cellulase enzymes of *G. natalis* may also be able to hydrolyse cellulose even in the presence of high levels of sugars from other dietary items such as fruits and seeds. High concentrations of glucose may be present in the stomach given that the stomach is the site of mastication and some enzymatic digestion while the midgut gland is the site of absorption.

By contrast, the cellulases of *D. hirtipes* had low activity and were susceptible to product inhibition, and overall cellulose hydrolysis in this species was not as efficient as that in *G. natalis*. Thus, *D. hirtipes* may require a diet that contains a greater amount of more readily digestible material to satisfy its energy requirements. This could explain why *D. hirtipes* prefers green to brown leaves while *G. natalis* shows no preference (Greenaway and Raghaven, 1998). Green leaves contain more digestible components such as cellular proteins, storage carbohydrates and lipids and less cellulose than brown leaves (Greenaway and Raghaven, 1998).

pH optima for cellulase activity

The activities of the cellulases examined were maintained across quite a broad range of pH (Table 1) but with maximal activities in the pH range 5.5–7. As the measured pH of the digestive juices of the study species (6.69±0.03 for *G. natalis* and 6.03±0.04 for *D. hirtipes*) fell within this range, the cellulase enzymes will operate at or near their maximal activity. EG and β-1,4-glucosidase from insects and the crustacean *Cherax quadricarinatus* had similar pH maxima, ranging between pH 4 and 6 (Watanabe et al., 1997; Tokuda et al.,1997; Table 4). The pH values of the digestive juices of *G. natalis* and *D. hirtipes* were similar to the pH of the gut of the herbivorous isopod *Porcellio scaber* (pH 5.5–6.5; Zimmer and Topp, 1997). Endo-β-1,4-glucanase from both *G. natalis* and *D. hirtipes* exhibited additional activity peaks at pH 9 (Table 1), however this is considered to be an artefact of the assay and would not occur *in vivo*.

Tannins inhibit digestive enzymes by binding to the protein and precipitating it. They precipitate more protein under acid than under alkaline conditions, and certain insects [e.g. the cricket *Telleogryllus commodus*, the New Zealand grass grub (*Costelytra zealandica*) and the gypsy moth (*Lymantria dispar*)] have highly alkaline guts (pH range 8–11) to counteract the precipitation of enzymes by dietary tannins (Cooper and Vulcano, 1997; Briggs and McGregor, 1996; Schultz and Lechowicz, 1986). The foregut fluid from *G. natalis* was slightly more alkaline than that of *D. hirtipes*, but the small difference is unlikely to significantly alter the effects of dietary tannins on the digestive enzymes of the two species as both pH values fell within the acid range.

Table 4. *Properties of endo-β-1,4-glucanases and β-1,4-glucosidases from various invertebrates*

	Endo-β-1,4-glucanase		β-1,4-glucosidase			
Species	pH optimum	K_m (mg ml^{-1} CMC)	pH optimum	K_m (mmol l^{-1} cellobiose)	K_i (mmol l^{-1})	Reference
Reticulitermes speratus (termite)	6	1.83, 1.48				Watanabe et al. (1997)
Nasutitermes takasagoensis (termite)	5.8	8.7				Tokuda et al. (1997)
Panesthia cribrata (cockroach)		9.4, 6.8		2.4, 13.8	0.33	Scrivener and Slaytor (1994)
Geoscapheus dilatatus (cockroach)		8.7, 4.6, 7.6				Scrivener et al. (1997)
Erinnyis ello (cassava hornworm)			6.5	2±0.2	0.0009	Santos and Terra (1985)
Locusta migratoria (locust)			5.5	1.95		Morgan (1975)
Cherax quadricarinatus (decapod crustacean)	4–5					Xue et al. (1999)
Gecarcoidea natalis (decapod)	5.5–7	3.03	4–7	5.84	11.12	Present study
Discoplax hirtipes (decapod)	5.5–7		4–7	4.23	4.53	Present study

CMC, carboxymethyl cellulose. K_i is the inhibitory constant of glucono-D-lactone on β-1,4-glucosidase.

Hemicellulase activities

Digestive juices from the gecarcinid land crabs *G. natalis* and *D. hirtipes* hydrolyse laminarin, lichenin and xylan and so contain activities of the hemicellulases laminarinase, licheninase and xylanase. Given that cellulose, laminarin, lichenin and xylose are carbohydrate polymers with sugar units joined by β-glycosidic bonds, it is possible that only one or two multicatalytic enzymes may catalyse all the reactions rather than multiple enzymes each catalysing one reaction. Exactly how many β-glycohydrolases are present in the digestive juice of the gecarcinid crabs is unknown and requires further investigation.

The respective activities of β-glycohydrolases in the study species were laminarinase > licheninase > xylanase > total cellulase. Consequently, assimilation of the different hemicelluloses and cellulose would be expected to follow a similar rank order, notably laminarin > lichenin > xylan > cellulose (Tables 2, 3). Thus, in diets containing equal amounts of hemicellulose and cellulose, both *G. natalis* and *D. hirtipes* would be expected to gain more carbohydrate from hemicellulose than cellulose. The high activities of laminarinase in the gut fluid of *G. natalis* and *D. hirtipes* indicate that they are particularly efficient in the hydrolysis of laminarin (Table 3). Laminarin is the chief food reserve of algae (Vonk and Western, 1984), and both species have been observed to scrape algae from rocks, soil and tree root buttresses (S.M.L., personal observation). Mixed β-D-glucans are a major component of cereals and grasses (Terra and Ferreira, 1994; McCleary, 1988). Gecarcinid crabs are able to utilise β-D-glucans since both *G. natalis* and *D. hirtipes* possess moderate licheninase activity (Table 3). *D. hirtipes* is better able to assimilate lichenin than *G. natalis* since its digestive juice has a higher licheninase activity (Table 3). *G. natalis* and *D. hirtipes* possess low xylanase activities and will only be able to hydrolyse xylan slowly.

Differences in assimilation coefficients for hemicellulose reported previously for the study species (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998) cannot be interpreted in terms of hemicellulase activities measured in this study, as the types of hemicellulose present in *Ficus* and *Erythrina* leaves are not known.

Differences in cellulase activities and properties may explain differences in cellulase assimilation and dietary preferences

G. natalis had a higher cellulose assimilation efficiency than *D. hirtipes* when fed a diet of brown fig leaves (43% and 21%, respectively; Greenaway and Linton, 1995; Greenaway and Raghaven, 1998), and the current study confirms that *G. natalis* hydrolyses cellulose more effectively than *D. hirtipes* since it has higher total cellulase activity. In addition, the β-1,4-glucosidase from *G. natalis* is resistant to product inhibition and consequently it can hydrolyse cellulose despite the presence of high levels of glucose from cellulose hydrolysis or simple sugars from other dietary items such as fruit. This property may also be indicative of resistance of the enzyme to the effects of dietary tannins.

Origin of the cellulase and hemicellulase enzymes

Cellulase and hemicellulase enzymes may be either produced endogenously or by microorganisms within the gut. Endogenous production is possible in the study species, given that other invertebrate groups such as insects, molluscs and nematodes possess a gene for EG and synthesize this enzyme endogenously (reviewed by Watanabe and Tokuda, 2001). The origin of cellulases in gecarcinid crabs is investigated in a subsequent manuscript.

The authors would like to thank N. McDonald and S. Raghaven for their invaluable technical assistance. This work was supported by Australian Research Council grants to P.G.

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